CONSTITUTIVE NEUROGENESIS IN THE ADULT BRAIN OF THE BEARDED DRAGON AND CORN SNAKE

Master's Thesis

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Tiivistelmä – Referat – Abstract

Reptiles have long been studied in search of the mechanisms behind neuronal regeneration. This thesis delves into the regenerative areas of two emerging model species to the field of regenerative research: *Pogona vitticeps* (bearded dragon) and *Pantherophis guttatus* (corn snake). This fluorescent immunohistochemical study maps out and compares the constitutive proliferative zones in these two species to better define the focus of future comparative neurodegenerative experiments. A BrdU pulse chase experiment in conjunction with PCNA reveals proliferative zones in the lateral ventricular ependyma of both species. Stem cell niches were found in the ependymal lining adjacent to the medial cortex and dorsal ventricular ridge in both species, however, the nucleus sphericus ependyma was an active proliferative zone only in *Pantherophis*.

Imaging of further markers in this study support the findings of the pulse chase experiment. High levels of the stem cell marker Sox2 was found in lateral ventricular ependymal cells in both species. The glial marker GFAP reveals a highly ordered array of radial glia in the cortical areas of *Pogona*, which is significantly reduced or absent in *Pantherophis*. And lastly the neuronal marker HU was found in the same cells that were BrdU positive and had migrated a short distance from the proliferative zones, which shows that the proliferative areas in the lateral ventricular lining do indeed produce neurons.

The BrdU and PCNA marked cells were quantified in both species, and a brief comparison between the species showed that *Pogona* had a significantly higher number and concentration of proliferative cells in the proliferative zones than *Pantherophis*. Scattered BrdU positive cells that were neither neuronal nor positive for any other marker were also found scattered throughout the parenchyma of *Pogona*, and these cells remain uncharacterized. Differences between these two species are not surprising, as lizards are known to have better regenerative capabilities than snakes, however, more comparative research between these species is needed to gain further insight into the mechanisms behind their contrasting regenerative capabilities.

Avainsanat – Nyckelord – Keywords
Reptiles, neurogenesis, regeneration, constituve regeneration, neuronal stem cells, corn snake, bearded dragon

Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors
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Säilytyspaikka – Förvaringställe – Where deposited

Muita tietoja – Övriga uppgifter – Additional information

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BrdU Bromodeoxyuridine (5-bromo-2'-Deoxyuridine), combines to DNA during replication instead of uridine.

CNS Central nervous system

GFAP Glial fibrillary acidic protein, an intermediate filament expressed in astrocytes, ependymal cells and radial glia

Hu Also known as ELAV-like protein, a protein found only in neurons in the CNS.

PCNA Proliferating Cell Nuclear Antigen, a protein needed for DNA replication and proliferation marker.

Sox2 Sex Determining Region Y box 2, a transcription factor important for maintaining self-renewal capabilities

PBS Phosphate buffered saline.

Brain areas:

OB olfactory bulb

MCX medial cortex

DCX dorsal cortex

LCX lateral cortex

DVR dorsal ventricular ridge

Lat V lateral ventricle

Sp septum

STR striatum

NS nucleus sphericus

Th thalamus

OpT optic tectum

Ce Cerebellum

Teg tegmentum

3rd V 3rd ventricle

Med medulla

1. Introduction

1.1. Background

Brain injuries in humans are notoriously difficult to heal, and often result in permanent brain damage. Neurodegenerative diseases and brain damage from strokes are increasingly common as the population ages and life expectancies increase, but cures for these ailments as of yet do not exist. Therefore, it is more important now than ever to find new ways to treat damage to the central nervous system (CNS). Research on humans and other mammals only takes us so far in understanding the full potential of the regenerative process, and studying animals that can regenerate lost brain tissue can widen our understanding of this process. Comparing the mechanisms behind regeneration in species that can heal brain damage, to the same mechanisms in species that do not have this ability, can help us gain insight into what makes it possible to reverse neuronal damage in one species and not another. When we understand the processes and conditions that facilitate regeneration, we may be able to recreate these regenerative capabilities in humans as well.

Lizards are a group of animals that is well known for its regenerative capabilities. In addition to being able to regenerate lost limbs, many of them are known to be able to regenerate damage to brain tissue. Proliferation and brain tissue regeneration has been studied in many species such as *Gallottian galloti* (Delgado-Gonzalez et al., 2011; Garcia-Verdugo et al., 1986), *Tarentola mauritanica* (Pérez-Cañellas & García-Verdugo, 1996), *Tropidurus hispidus* (Marchioro et al., 2005) and *Anolis carolinensis* (Duffy et al., 1990). The most studied lizard species is *Podarcis hispanica* which is able to regenerate the medial cortex (MCX) after lesions that have almost fully destroyed it (Font et al., 1997; C. Lopez-Garcia et al., 1988; Carlos Lopez-Garcia et al., 2002; Molowny et al., 1995; Ramirez-Castillejo et al., 2002). A review by Font et al. (2001) collected studies on proliferation in reptiles, and though there are variations in proliferation rates in different telencephalic areas, the MCX lateral ventricular ependyma is a proliferative hot spot in all the species mentioned.

In reptiles the MCX is homologous to the human hippocampus, where neuronal stem cells have also been found in humans. Humans, therefore, have neuronal stem cells in similar areas as lizards, but cannot regenerate brain tissue efficiently. This differential capacity of existing stem cells to produce new neurons capable of normal function and integration into existing neuronal networks after injury raises an array of interesting research questions, which include but are not limited to: why CNS regeneration is at times possible but not in all brain tissue, classification of cells that produce neurons in the matured CNS and where they are located, what signals promote or restrict regeneration and finally can these signals be artificially induced to promote regeneration in other species.

Currently research indicates that neuronal stem cells and regeneration seem to be bound to a functional need for production of new neurons in the normal state (Alunni & Bally-Cuif, 2016; Ferretti, 2011). This means that stem cells are located in or connected to areas where new neurons are continually being replenished throughout the adult life. These stem cell niches house a permissive environment for stem cells to persist in adulthood, and proliferative zones are highly conserved in the vertebrate brain (Alunni & Bally-Cuif, 2016; Kaslin et al., 2008). According to multiple review articles (Alunni & Bally-Cuif, 2016; Emsley et al., 2005; Font et al., 2002; J. Kaslin et al., 2008) parallels can be drawn between sites of constitutive regeneration between species. The homologous reptilian MCX and mammalian hippocampus both receive new neurons that incorporate into the existing neural circuitry throughout adult life. The MCX in reptiles receives new neurons from the lateral ventricular lining directly below it, and the mammalian dentate gyrus produces new granule neurons to aid in learning and memory. The lateral ventricular lining in both mammals and reptiles produces neuroblasts that migrate to the olfactory bulb along the rostral migratory stream (RMS)to replenish the olfactory interneurons that are constantly being replaced. As these similar areas in multiple species are capable of producing new neurons in adults, it is interesting that neuronal regeneration is not more common. There must be different mechanisms at work to either hinder of facilitate neuronal regeneration in different species. One distinction between reptiles and mammals is the life-long continuous growth in size in reptiles but not in mammals, which creates contrasting needs for neuronal constitutional regeneration between species.

Reptiles grow throughout their lifespans, which is why they have a more widespread need for constitutive regeneration than mammals. Most of the constitutional regeneration occurs in sensory areas and areas related to memory (MCX / hippocampus) (Ferretti, 2011). Because of this need for new neuronal cells in multiple brain regions, the environment in these regions must permit axonal growth and neuronal integration. This environment is very promising in the search for possible medications that may help with permitting neuronal integration in other species. Mammals stop growing before adulthood, and therefore, do not need to keep up this permissive state. It may be more beneficial to sacrifice some plasticity for stability in the complex mammalian brain (Kaslin et al., 2008). Though the mammalian brain produces new neurons, they do not integrate into existing neural networks after brain damage, as they do in many reptiles.

As with lost limbs, the absence of scarring in brain tissue is evident in non-mammalian vertebrates (Kaslin et al., 2008). The mammalian brain creates a glial scar that prevents the newly formed neurons from reaching their destinations and making the necessary connections to successfully integrate into the neural network. This physical blockage alongside differences in inflammation patterns after brain insult affect reactional regeneration differentially between species (Alunni & Bally-Cuif, 2016). Not only are there differences in the normal state, but also in the aftermath of a brain injury.

The future objective is to see differences between species that can regenerate brain tissue and humans who comparatively cannot. There can, however, be many confounding factors that stem from differences in brain anatomy and function between species this far from each other in the evolutionary tree. For the purposes of this first study, it is better to compare two reptiles. One lizard species with good regenerative capabilities: *Pogona vitticeps* (bearded dragon), and the other a snake with less spectacular regenerative capabilities: *Pantherophis guttatus* (corn snake). Not many studies on regeneration have been carried out on snakes, but it is our understanding that their regenerative capabilities and constitutive neurogenesis (Eymann et al., 2019) are less than that of lizards. This understanding is backed up by reviewed leasonal studies on the optic nerves of different reptile species (Rodger & Dunlop, 2015). By comparing species that are comparatively close on the evolutionary

tree, many of the confounding factors can be taken out of the equation. With similar brain structure it is easier to compare the location and density of stem cells in different brain regions, and any differences in these may be the reason for the disparity in regenerative capabilities. With a better understanding of differences between reptiles, it will be easier to interpret the relevant differences between species that are further diverged in the evolutionary tree.

1.2. Aims of this study

This study has two aims: (1) to map out the putative stem cell niches in the two emerging model species *Pantherophis guttatus* (corn snake) and *Pogona vitticeps* (bearded dragon), and (2) to compare putative stem cell location and density between these two species during constitutive neurogenesis. The intent is to understand what is happening in the normal state in our species, when only constitutive regeneration is taking place. Previous research on constitutive regeneration has focused on the telencephalon of a small number of lizard species, with very little research on other brain regions, which is why this study will include sections from different areas throughout the brain. Differences in constitutive regeneration may reflect on reactive regeneration, which is triggered after brain insult. The function of this experiment is to assess whether there is a difference in constitutive regenerative areas between the two species to better define the focus of future comparative neurodegenerative experiments.

These are novel species in regenerative research, consequently there is no body of research on them to be drawn upon. Thus, background research is necessary to understand what is happening in these species. The advantage to using these new species is their relative availability, due to them being common pets, and the fact that genome data on these species is readily available. The first step towards the comparison of regenerative zones between *Pogona* and *Pantherophis* is mapping their brains and finding out which areas correspond between the species. Mapping the putative stem cell niches will be the main focus of this study.

2. Materials and methods

2.1. Animals

Four specimens in total were used for mapping out the proliferative zones of the species, two *Pantherophis guttatus* and two *Pogona vitticeps*. No more than two specimens of each species were used, because the aim of the experiment was to map where the constitutive proliferative zones are in the normal state, in order to spot areas of interest for subsequent experiments. Looking at two specimens from each species also helped rule out anomalous findings that may occur when looking at only one specimen and made it possible to carry out a BrdU pulse experiment. The specimens were 2-month-old juveniles from the Helsinki University animal facility.

Most of the presented samples were collected, embedded, and sectioned by other members of the research group, and the slides were taken directly from Nicolas Di-Poï's laboratory storage. One snake sample (D0) did not have coronal sections, therefore, the BrdU treatment, embedding, sectioning, and preparation of the slides for this individual was carried out by myself.

2.2. BrdU pulse-chase treatment

Each individual was given 80 mg/kg BrdU twice a day for one week. The bromodeoxyuridine (BrdU) was mixed into water (20 mg/ml) and given orally through a pipette. One snake and one lizard were sacrificed immediately after the 7-day pulse labelling treatment (T0 in figure1). Slides obtained from these individuals show where the cells are dividing. One snake and one lizard were then sacrificed one month after the BrdU treatment (T1 in figure1). Slides obtained from these individuals show possible migration of the cells that have divided during the BrdU treatment. The one-month time period was chosen based on previous trials of 15 days, one month, and

two months; and the one-month time period was deemed optimal for detecting slow-cycling stem cells in brain tissue.

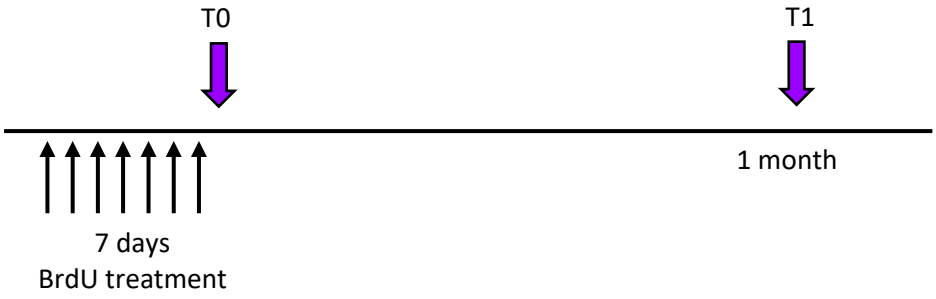


Figure 1. Timeline for BrdU pulse-chase treatment

The seven black arrows show the days of BrdU administration (pulse). The Purple arrows show when the animals were sacrificed. At T0 two animals were sacrificed directly after BrdU treatment, and at T1 two animals were sacrificed after one month of chase.

2.3. Embedding and sectioning

Following animal euthanasia, the brains were dissected from the skulls in 1 X PBS. The brains were dehydrated in methanol (series of washes in 30 % -> 50 % ->70 % -> 90 % methanol and 1 X PBS) and embedded in paraffin blocks for sectioning. Embedding was carried out using a Leica ASP200 embedding machine with a standard program (30 min x 2 + 1h in absolute ethanol, 30 min x 2 + 1 h in xylene, 3 x 1 h in paraffin).

The blocks were cut to 10 µm coronal slices using a microtome, and four consecutive sections were mounted to each charged adhesion slide using a hot water bath (+47°C). The slides were dried overnight in +37°C and the paraffin was melted again on hotplates (+70°C) for 10 seconds. The slides were then stored at +4°C.

2.4. Immunohistochemistry

Several different fluorescent immunohistochemical stainings were used to map out the proliferative zones of the two species. Primary antibodies (see figure 3 and table 1) were chosen to show proliferation, mark possible stem cells, and show neuronal differentiation of new cells. BrdU shows proliferation at the timepoint it was administered, as it incorporates into replicating DNA. Therefore, at T0 it identifies recently divided cells, and at T1 it identifies cells that have divided at T0. BrdU paired with proliferating cell nuclear antigen (PCNA), which marks cells that are proliferating at the moment of sacrifice, double labels cells that divided at T0 and divided again at T1 in the individuals sacrificed at T1. This treatment identifies double labelled cells as possible stem cells (section 1 in figure 3). BrdU runs throughout the different treatments as a marker for possible stem cells and their daughter cells and marks the same types of cells in each treatment except section 4 on each slide. The aim of the section 4 staining was to map out both Nestin and glial fibrillary acidic protein (GFAP) positive cells in these species. In hindsight it would have been better to use BrdU and GFAP in this section to see whether the stem cells are the same as GFAP positive radial glia. Sex determining region Y box 2 (Sox2) is a stem cell marker, so the BrdU positive stem cells should also be positive for Sox2 (section 3 in figure 3). Hu specifically marks neurons and can be found early in the differentiation process. Combining BrdU and Hu (section 2 in figure 3) shows whether the cells that have divided at T0 have differentiated into neurons at T1.

Slides with coronal sections were selected at 10 slide intervals throughout the brain, so that similar slides could be analyzed for all individuals: 16 slides for the snakes and 15 slides for the lizards (see figure2). The differing number of slides between the species is due to the lizard samples lacking the olfactory bulb. The 10-slide interval was maintained throughout in both species, except in the case of the olfactory bulb in the snake samples, which was taken at a similar location in both samples.

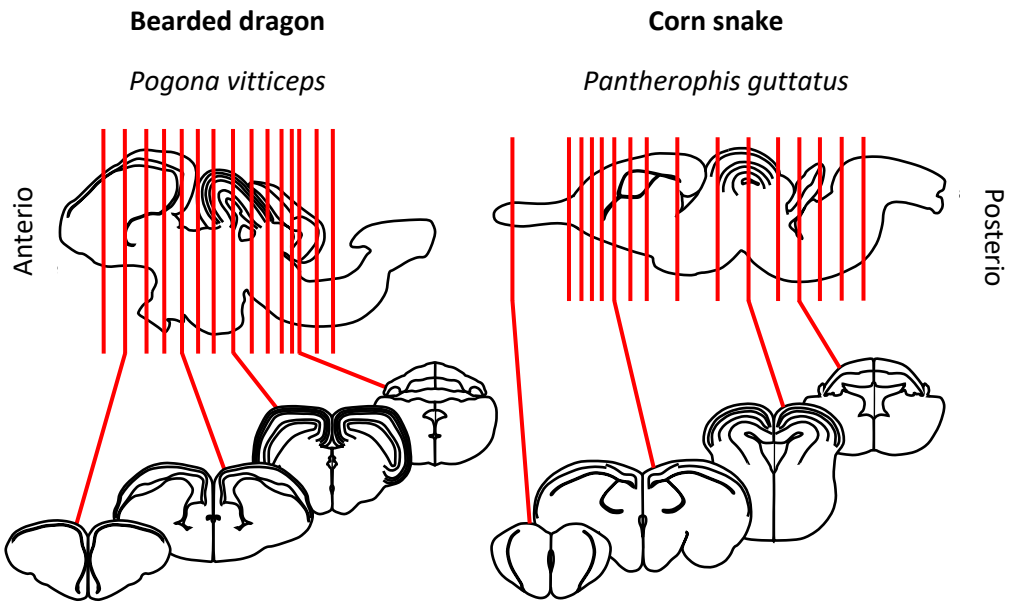


Figure 2. Slide selection and position

On the top left a sagittal section of a *P. vitticeps* brain and on the top right the same for *P. guttatus*. The red lines indicate cross sections at the points where the slides used were situated. A 10-slide interval was kept in all samples, with the exception of the *P. guttatus* olfactory bulb, which can be seen at the anterior end of the section. The lines represent the anatomical locations of the slides used for this experiment. They are not uniformly distributed along the sagittal section, because a different individual was the model for the sagittal sections, and the orientation of the sample in the paraffin block can make a difference in which areas may be more squished or elongated. The bottom section demonstrates a selection of the coronal sections used for the experiment.

A 3-day protocol was used for staining. First the paraffin was removed from the slides with a deparaffinization and rehydration series under a fume hood (xylene 2 x 10 minutes, 100% ethanol 2 x 2 minutes, 90% ethanol 2 x 2 minutes, 70% ethanol 2 x 2 minutes, 50% ethanol 2 x 2 minutes, and 1X PBS 2 x 2 minutes). Then antigen retrieval was performed using a citrate buffer (9 ml citric acid, 41 ml sodium citrate, and 450 ml distilled water titrated to pH 6,0 with NaOH). The citrate buffer was brought to a boil on a Bunsen burner in a 1L beaker. The slides were added to the buffer and kept at boiling point for 20 minutes. After heating, the beaker was placed in room temperature to cool down for 15 minutes, and then in an ice bath for further cooling for another 15-20 minutes. The slides were then washed in 1X PBS 3 x 10 minutes on a shaker.

The four sections on each slide were ringed with a wax pen before blocking, to prevent the different drops of antibody solution administered on each section accidentally merging. Blocking of non-specific binding was performed with 10% normal goat serum in 1X PBS for all sections. This was done using a humidifying chamber in room temperature for 1h. 50 µl of solution was used for each section on the slide.

Each of the four consecutive slices on each slide were given different antibody treatments (see figure 3). All antibodies were diluted in 1% bovine serum albumin and 1 X PBS. The antibody solutions had been previously tested to see which dilutions worked best, and those dilutions were used in this experiment (see table 1). After blocking, the slides were washed in 1 X PBS for 5 minutes on a shaker. The first set of primary antibodies was added to each section (PCNA, Hu, Sox2, and Nestin respectively as per figure 3). The slides were placed in a humidifying chamber and left to incubate overnight in +4 ˚C.

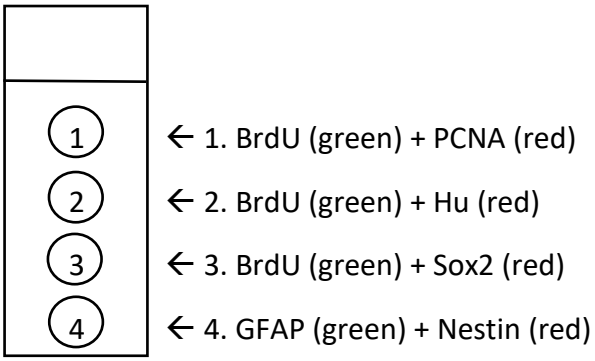


Figure 3. Treatments for the different sections on each slide

Each of the coronal sections marked 1-4 on each slide were given different antigen treatments shown by the arrows. All slides were treated the same way.

The slides were then washed in 1 X PBS 3 times for 5 minutes on a shaker to clear out the primary antibody solution. The secondary antibody solution was made in 1% bovine serum albumin and 1 X PBS using opaque tubes and the main light source turned off to avoid bleaching. The dilutions were based on the dilutions for the primary antibody, doubling the dilution. If a dilution of 1:200 was used for the primary antibody, a dilution of 1:400 was used for the secondary antibody. The first round of

secondary antibody was red Alexa Fluor 568 (see table 1). However, the red secondary antibody in snake T1 was Alexa Fluor 594, which performed equivalently to Alexa Fluor 568. 50 µl of the solution was placed on each section with the primary light source of the room switched off. The slides were then incubated for 1h in room temperature in a light shielded humidifying chamber. All subsequent steps after adding the light sensitive secondary antibody were performed shielding the sections from unnecessary light.

The second round of primary antibodies (BrdU and GFAP) was added after washing the unbound secondary antibody off in 1 X PBS 3 x 5 minutes on a shaker (see table 1). Everything was done the same way as in the first round of primary antibody, including overnight incubation at +4˚C. The only difference being light shielding and which antibodies were used.

The second round of secondary antibody was done exactly the same way as the first round of antibody, but this time using green Alexa Fluor 488 to differentiate from the red used previously (see fig 3). After the 1h incubation at room temperature, the slides were once again washed in 1 X PBS 3 x 5 minutes. The slides were then covered with glass, using Sigma Fluoroshield with DAPI.

Table 1. Antibodies for immunohistochemistry

Primary antibody	**Species raised in**	**Dilution**	**Secondary antibody**
BrdU	Rat	1:200	Goat-anti-rat Alexa Fluor 488 (green)
GFAP	Mouse	1:200	Goat-anti-mouse Alexa Fluor 488 (green)
PCNA	Mouse	1:200	Goat-anti-mouse Alexa Fluor 568 (red)
Hu	Mouse	1:600	Goat-anti-mouse Alexa Fluor 568 (red)
Sox2	Rabbit	1:200	Goat-anti-rabbit Alexa Fluor 568 (red)
Nestin	Rabbit	1:200	Goat-anti-rabbit Alexa Fluor 568 (red)

2.5. Imaging and cell count

The slides were scanned at the Biomedicum Genome Biology Unit with a 3DHistech Panoramic 250 FLASH II digital slide scanner at 40X magnification. The scanning produced images with three layers, with each layer representing one treatment (Alexa 568 red, Alexa 488 green, and DAPI blue).

Only section 1 from each slide was quantified. The BrdU, PCNA, DAPI, and BrdU-PCNA double labelled cells were counted from these sections. The other sections from each slide were scanned, but not quantified. Only qualitative analysis of these treatments was made to shed more light on the proliferative areas found from BrdU and PCNA analysis.

The cells were counted separately for the main brain regions and areas of interest using the image processing software ImageJ. The images were exported from the HistoQuant viewing software in Extended TIFF format, with each layer in a separate image. All layers were opened using ImageJ. Specific brain regions were cut from the full images for analysis, and the same selections copied to each layer. Moving through the different brain regions in each image, the previous selection was completely removed from the full image, so that no overlap was possible.

A cell count was performed on each treatment separately, and double labelled cells were counted separately. Cells were counted using the threshold function paired with the watershed function to separate merged dots for cells that are close to each other. Thresholds were set separately for each treatment and was only changed to better represent what could be seen on each slide if background levels varied. The actual count was performed using the analyze particles function on ImageJ on the thresholded images turned to binary. Cutoff points for particle size were 100-1500. Particles larger or smaller than this size were not cells and were discounted from the cell count. Any obvious blood vessels were removed from the images before thresholding, as this method detected false positives from them. The cells were very close to each other in some areas, such as the ventricular lining, and the threshold treatment did not work in these areas, so the cells were counted manually.

Double labelled cells were counted by creating colored masks of the binary layers used for the cell count, one treatment in blue and the other in red. This way the threshold and the cell count are exactly the same as in the cell count of the separate treatments. When the colored masks were merged, the double labelled cells showed up in magenta, and were counted manually from each section. The data from the cell count was recorded in an excel sheet with each slide and brain area separately.

3. Results

3.1. Mapping the proliferative zones of the bearded dragon and corn snake

3.1.1. Pogona Vitticeps

3.1.1.1. BrdU and PCNA in different brain regions of Pogona vitticeps

BrdU and PCNA markers show where proliferation takes place in the central nervous system, BrdU showing which cells were proliferating at the time of BrdU administration and PCNA which cells were proliferating when the animal was sacrificed. At T0 most of the proliferating cells are double labelled, as the time between BrdU administration and sacrifice is brief. Therefore, double labelling with these markers account only for proliferation in general at T0. At T1, however, there is one month between BrdU administration and sacrifice, therefore, double labelled cells indicate which cells have divided at T0 and were dividing again at T1. These cells are slowly cycling putative stem cells and the main focus of this experiment.

During development the brain begins to form the different major brain regions: prosencephalon, mesencephalon, and rhombencephalon. These regions then further

divide into the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon. For my division in this experiment I have separately counted labelled cells for the telencephalon, diencephalon, mesencephalon, cerebellum, and rhombencephalon (see figure 4). The separation was completed in this manner due to practical reasons, as the division between the tegmentum of the metencephalon and the beginning of the myelencephalon are difficult to distinguish in the sections. These two areas have been treated as one and named the rhombencephalon from developmental nomenclature. The cerebellum, however, has been processed separately, as it is easy to see the delineation between it and the metencephalic tegmentum.

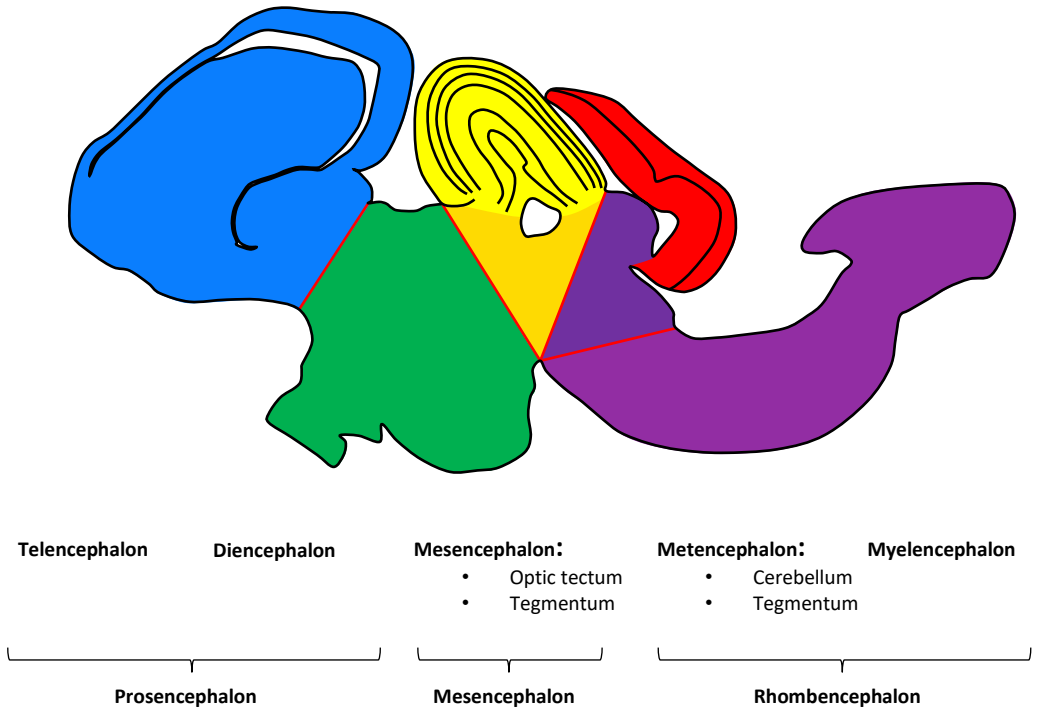


Figure 4. The major brain regions in *Pogona vitticeps*

The major brain regions can be seen divided into colors that correspond to the following figures for cell counts in these regions. The telencephalon can be seen in blue, the diencephalon in green, the mesencephalon in yellow, the cerebellum in red, and finally the rhombencephalon in purple. During development the prosencephalon divides into the telencephalon and the diencephalon, and the rhombencephalon divides into the metencephalon and myelencephalon (these regions can be seen divided by the red lines). The mesencephalon is made up of the optic tectum (light yellow) and the tegmentum (dark yellow). The metencephalon is made up of the cerebellum (red) and the tegmentum

(dark purple). The rhombencephalon without the cerebellum has been processed as one area in my data (dark purple and light purple).

There is proliferation throughout the Pogona brain, but most of the proliferation is seen in the telencephalon (see figures 5-7). The telencephalon has the highest number of proliferating cells, with most of the PCNA positive cells from both time points found in a highly proliferative zone in the lateral ventricular lining. The ventricular lining in other areas, however, is not proliferative in the normal state, and no dividing cells were found in the ependyma of these ventricular regions. The remaining proliferating cells are sparsely dispersed throughout the parenchyma in the other brain regions, with no specific regions of proliferation.

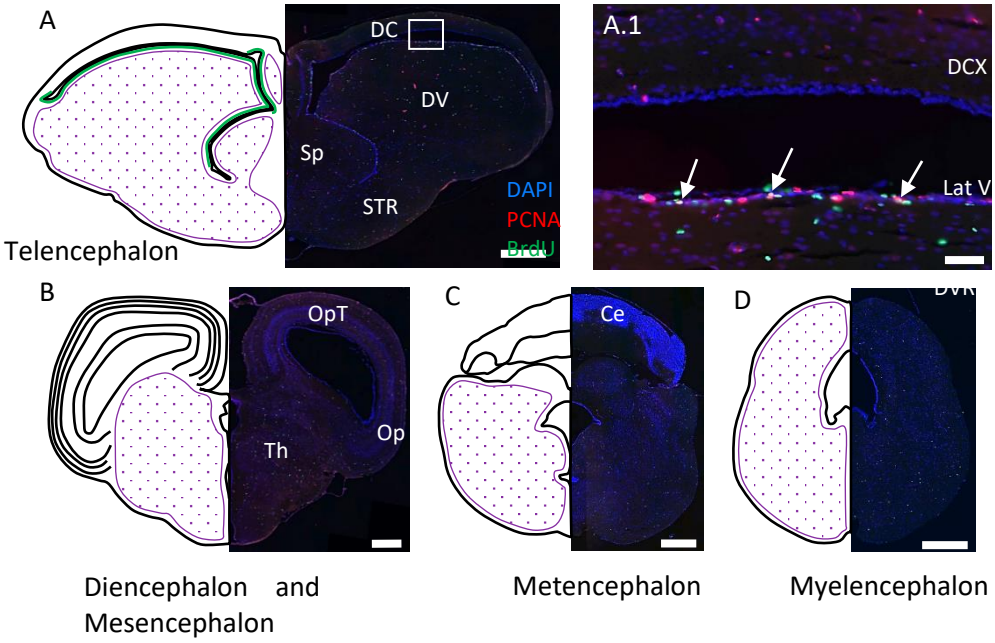


Figure 5. Proliferative areas throughout the *Pogona vitticeps* brain at T1

This figure shows how the proliferating cells are distributed throughout the brain at T1. Selected sections from all the different brain regions are shown with the right half of the image as the microscope view and the left side as an illustration of the proliferating regions. In the microscope images the total amount of cells can be seen in blue (DAPI), BrdU in green, and PCNA in red. In the illustrations the purple dotted area depicts scattered PCNA+ cells and BrdU+ cells, but no or very few double positive cells. The green line represents a hotspot for proliferation, with BrdU+, PCNA+, and double labelled cells. **A.** The ependyma of the lateral ventricles is a proliferative hotspot with possible stem cells double positive for BrdU and PCNA. There are proliferating cells, but very few putative stem cells dispersed throughout the parenchyma. The white box demonstrates the area from which A.1. is enlarged. **A.1.** A

close up of the lateral ventricular region showing abundant proliferation and double labelled stem cells (white arrows). **B.** The diencephalon (optic tectum) and mesencephalon showing proliferation throughout the mesencephalic parenchyma, with less proliferation in the optic tectum. There is no proliferation at all in the ependyma of any of the other ventricles, but the dispersed parenchymal proliferating cells can be seen throughout the brain (C. and D.). **C.** The metencephalon showing the cerebellum and tegmentum. The cerebellum has fewer proliferating cells than the tegmentum. **D.** The myelencephalon.

Scale bars 500µm for all except A.1. 50µm.

The cell counts reveal that 80 % of the total number of counted PCNA+ cells are found in the telencephalon at T1 (see figure 6). The other brain regions account for 20% of the PCNA+ cells as follows: diencephalon 4%, mesencephalon 8%, cerebellum 3%, and rhombencephalon 6%. Not only is the number of dividing cells larger in the telencephalon, but the proportion of dividing cells from the full cell count of the area is higher as well, with almost 2% of all cells in a proliferative state at T1. The proportion of dividing cells in all the other areas is smaller than 0,35% of all cells.

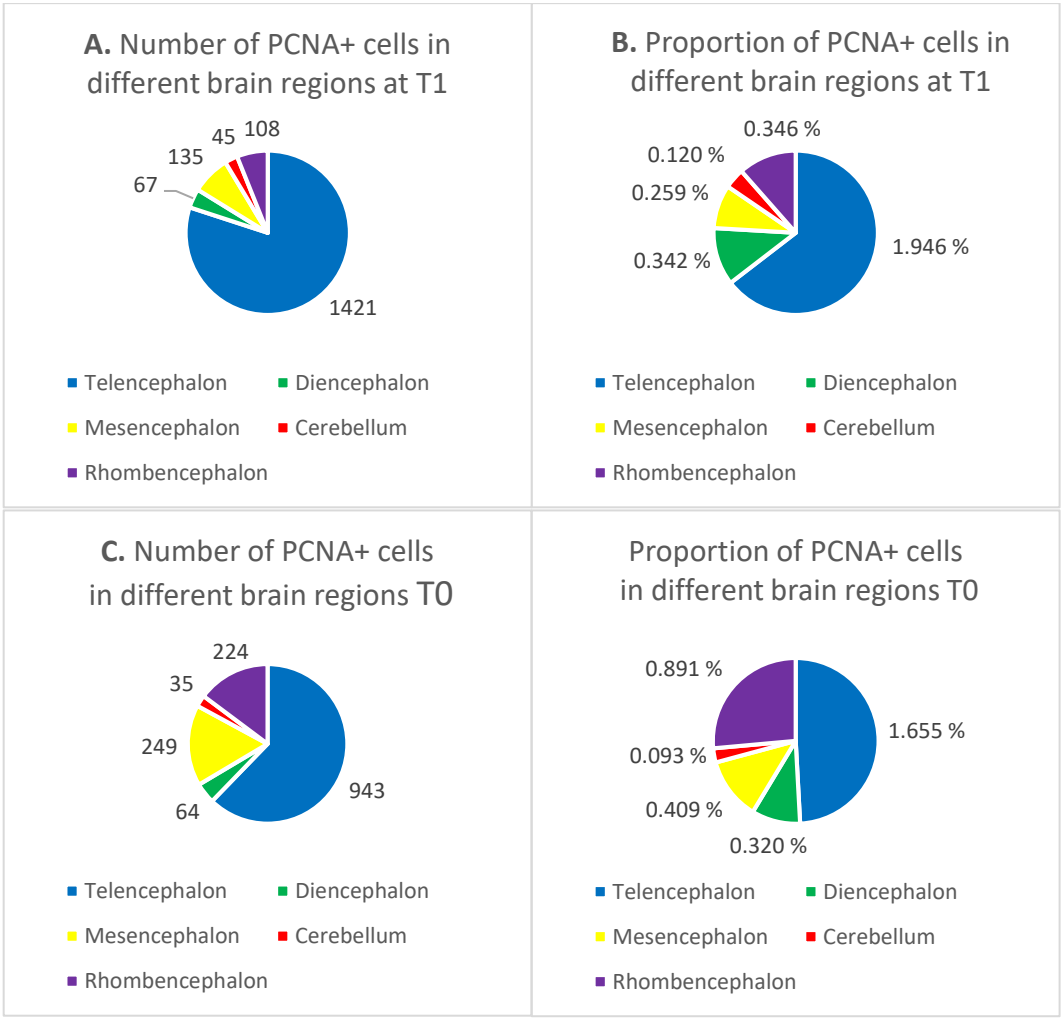


Figure 6. Proliferation in different brain regions in *Pogona vitticeps*

A. The total number of PCNA positive cells in different brain regions at T1.

B. The proportion PCNA positive cells out of the full cell count of each region at T1.

C. The total number of PCNA positive cells in different brain regions at T0.

D. The proportion PCNA positive cells out of the full cell count of each region at T0.

The amount of proliferation at T0 shows a similar story, with most of the proliferation occurring in the telencephalon. However, the amount and proportion of PCNA+ cells is more distributed throughout the different major brain regions. The telencephalon still has the highest proportion of proliferating cells at 1,655%, with the rest of the areas below 0,9%. This difference may be due to variation between individuals or the slight difference in age between T0 and T1.

Though there is proliferation throughout the *Pogona* brain, prospective stem cells can only be found in the lateral ventricles of the telencephalon (see figure 7). This finding is congruent with findings in other species of lizard.

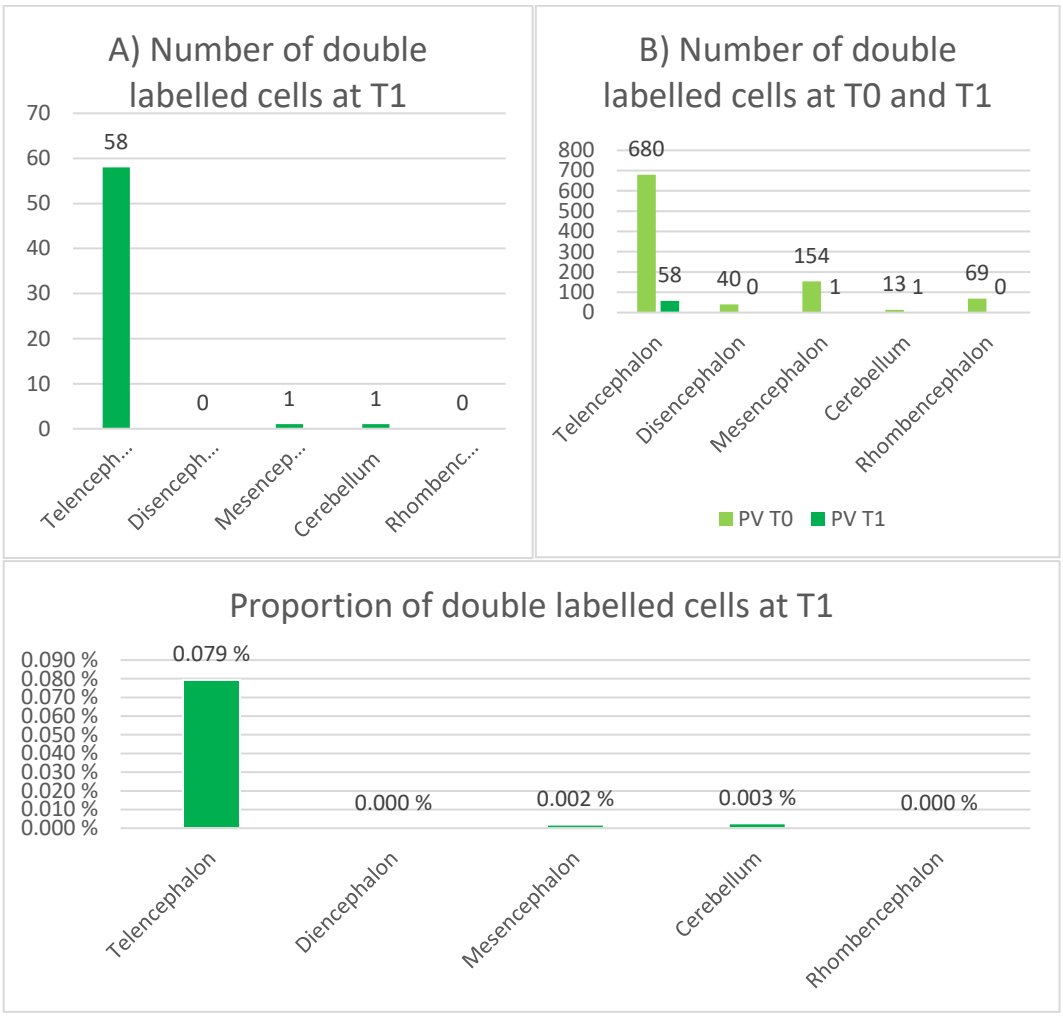


Figure 7. Distribution of putative stem cells in different brain regions in *Pogona vitticeps*

This figure shows the number and proportion of cells that are double labelled with BrdU and PCNA in the different brain regions. These cells have divided at T0 and were dividing again at T1 and are possibly stem cells. **A.** how many double labelled cells were found in the different brain regions at T1, showing the number of possible stem cells in the different brain regions. The stem cells are confined to the telencephalon. **B.** the change of the number of double labelled cells from T0 to T1. At T0 the number of BrdU and PCNA double labelled cells indicates how much proliferation is happening in the different brain areas. At T1 we have the number of possible stem cells shown separately in A. **C.** the percentage of all cells in each brain region that are double labelled.

The number of prospective stem cells that can be seen at T1 is only 58 out of a total of 73011 cells counted from the sections of the telencephalon. This comes up to 0.079% of the total telencephalic cell count being stem cells. Even a small amount of slowly cycling stem cells can keep up with the demands of constitutive regeneration, as they do not deplete themselves with multiple divisions, but run through the cell cycle repeatedly over an extended period of time.

3.1.1.2. BrdU and PCNA in different regions of the Pogona vitticeps telencephalon

The brain region with the most proliferation is the telencephalon, with most of the proliferation happening in the lateral ventricular lining (see figure 8). The lateral ventricular lining is also the area with the most prospective stem cells (double labelled with BrdU and PCNA at T1). This area, therefore, needs a separate breakdown as to where the proliferation is taking place.

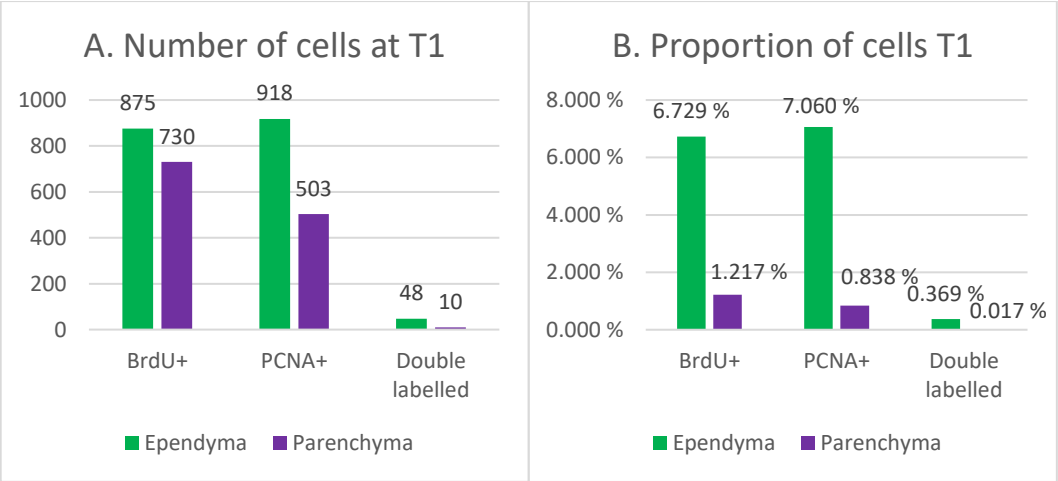


Figure 8. Proliferation in the lateral ventricular ependyma compared to telencephalic parenchyma in *Pogona vitticeps*

This figure shows the number (A.) and proportion (B.) of labelled cells from the full cell count of the lateral ventricular lining (green) compared to the telencephalic parenchyma (purple) at T1. BrdU shows proliferation at T0, PCNA shows proliferation at T1, and BrdU-PCNA double labelled cells show possible stem cells. Both the number and proportion of proliferating cells is higher in the ventricular ependyma than the surrounding parenchyma. Almost all of the possible stem cells were found in the ventricular

lining, with only 10 or 0.017 % in the surrounding parenchyma. This comparison confirms the lateral ventricular ependyma as a proliferative hotspot.

The telencephalon parenchyma and ependyma have BrdU+ and PCNA positive cells in the high hundreds, with the ventricular ependyma still having a few hundred more than the parenchyma. The volume of the ependyma is much smaller than the parenchyma, therefore, the proportion of labelled cells is a much more representative expression of cell density. In graph B. the difference between proportions of labelled cells is even more pronounced than in the number in graph A. (figure 8). The Ependyma has a much denser population of proliferating cells than the surrounding parenchyma. Only one sixth of the double labelled cells can be found in the parenchyma, with the other five sixths nestled in the proliferative zones of the lateral ventricular ependyma, and all of the double labelled parenchymal cells were located close to the ventricles. These double labelled cells are our putative stem cells.

The distribution of prospective stem cells is not uniform throughout the lateral ventricles (see figures 9 and 10). The MCX and dorsal ventricular ridge (DVR) ependyma are the most active, while some stem cells can be found in the septal (Sp) and striatal (STR) ependyma. The density of stem cells in the MCX ependyma is the highest in the entire brain (1,22 % of the total cell count of the area). The DVR ependyma has much more surface area than the MCX, and therefore, a lower proportion of active stem cells may have a large impact on regenerative capabilities. The total number of stem cells in the MCX and DVR is similar (12 and 15 respectively, see figure 9 C). There are virtually no stem cells seen in the dorsal cortex (DCX) ependyma.

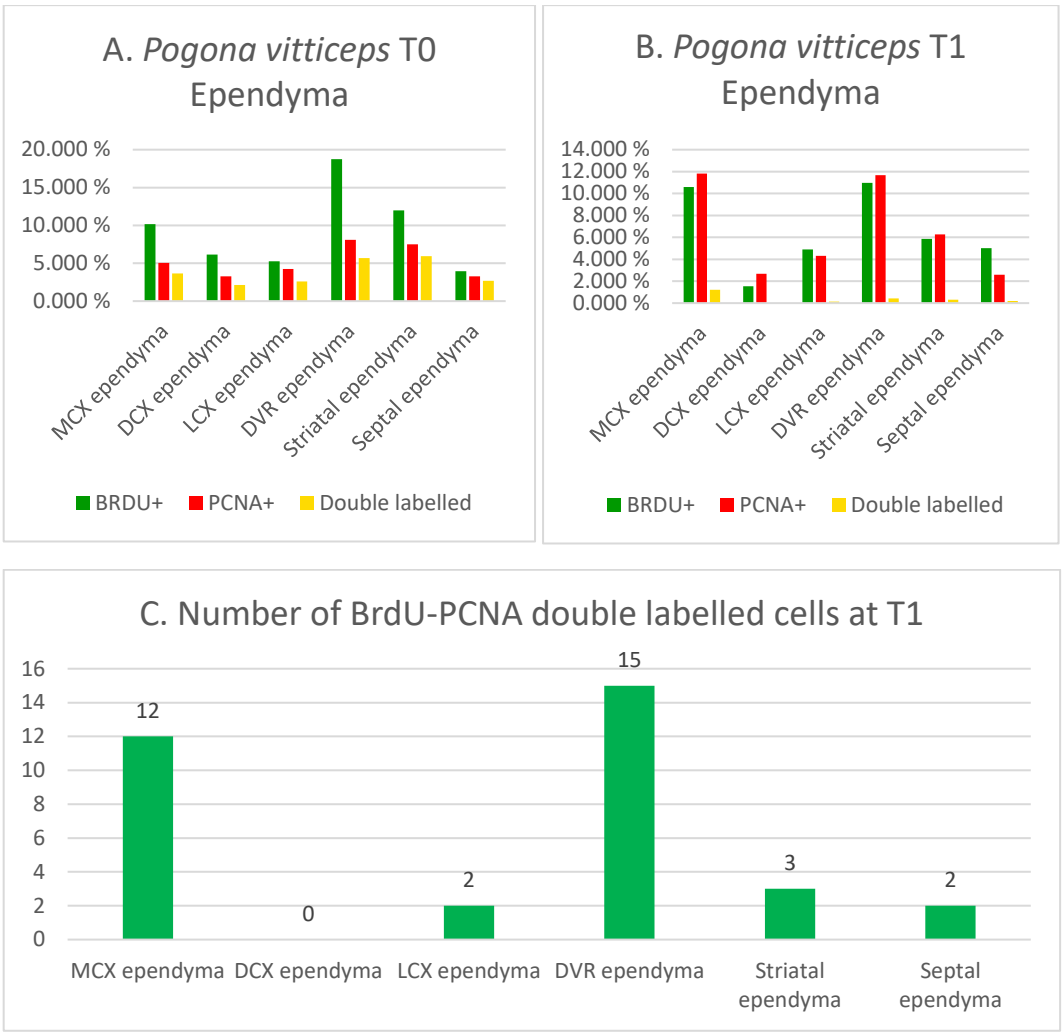


Figure 9. Proliferation in the lateral ventricular ependyma of *Pogona vitticeps*

A. Proportion of proliferating cells in the lateral ventricular ependyma at T0. At this time point all markers are indicative of proliferation, which can be seen throughout the ventricular lining. **B.** Proportion of proliferating cells at T1. PCNA indicates proliferation, BrdU indicates which cells divided at T0, and double labelled cells indicate possible stem cells. **C.** Number of double labelled cells in the different ependymal regions of the lateral ventricle at T1.

Proliferation can be seen in all areas of the lateral ventricles (figure 10). The MCX, DVR, and striatal regions have the highest density of proliferating cells at both timepoints, with the DCX left with the least dense population of dividing cells and no prospective stem cells. All other areas had a small number of prospective stem cells, of which the MCX and DVR had the highest number. The MCX had the highest density of prospective stem cells. The areas with a high level of proliferation and stem cell activity are the proliferative zones of the lateral ventricular ependyma.

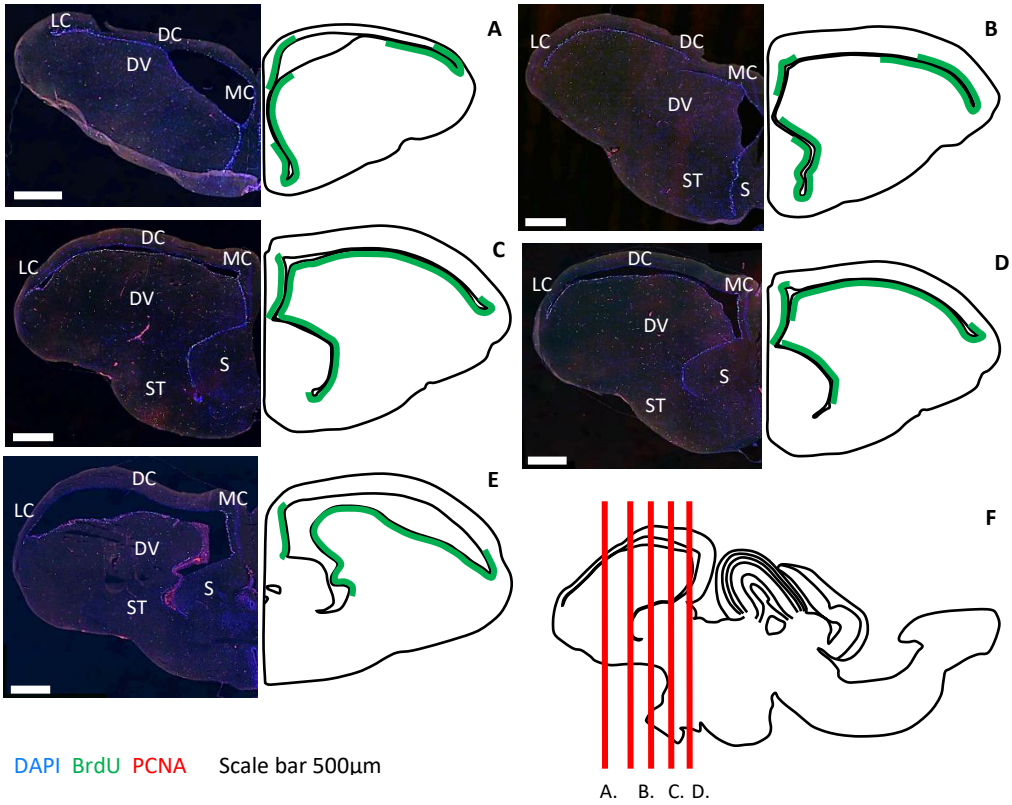


Figure 10. The lateral ventricular proliferative zone in *Pogona vitticeps*

A.-E. Coronal sections throughout the telencephalon at T1 showing the proliferating areas of the lateral ventricular lining. The left half of each section is a microscope image showing DAPI (blue), BrdU (green), and PCNA (red). The right half of each section is a schematic drawing showing the sections of the lateral ventricular ependyma which have high levels of proliferation (green line). **F.** Sagittal view of the full brain showing where each section is located.

The proliferative zone of the lateral ventricles is not continuous (see figure 10). Starting from the anterior end of the telencephalon the proliferative zone covers the MCX, septal, and striatal ependyma and the lateral tip of the ventricle going deep into DVR and LCX territory (panels A. and B.). Moving towards the posterior sections, the lateral cortex (LCX) proliferative zone shrinks to cover only the very lateral tip of the ventricle. The septal proliferative zone disappears, and the striatal proliferative zone shrinks progressively to leave only the MCX and DVR ependyma as the only proliferative zones in the posterior telencephalon.

3.1.1.3. Sox2 expression pattern in Pogona vitticeps

Sox2 is a transcription factor that maintains pluripotency in cells and is important in neuronal differentiation. It is highly expressed in neural progenitor cells (Zhang & Cui, 2014) and is, therefore, a good and highly conserved stem cell marker in the central nervous system. However, a low level of background is expected with this marker, as it is merely down regulated and not completely absent in neuronal cells. The difference in intensity is enough to differentiate between high levels in stem cells and low levels in neuronal cells.

High levels of Sox2 can be seen throughout the ventricular lining of all ventricles, except the tectal ventricles and the dorsal part of the lateral ventricle. Some, but not all of the BrdU labelled cells in the ventricular ependyma were also positive for Sox2 (see figure 11 A.1. and A.2.). The double positive cells confirm that some of these BrdU labelled cells could in fact be stem cells.

None of the BrdU positive cells found outside of the ventricular lining were Sox2 positive. This finding points to the parenchymal cells being something other than stem cells and indicates that these cells are likely not neuronal.

High expression of Sox2 can also be seen in the large neurons of the trochlear and oculomotor nuclei as well as the inferior reticular system. Some expression of this marker is expected, as low levels of Sox2 are present in neuronal cells, but these large cells seem to have higher expression of Sox2 than other neurons. None of these cells are positive for BrdU, and the HU staining confirms them to be neuronal cells (see figure 13).

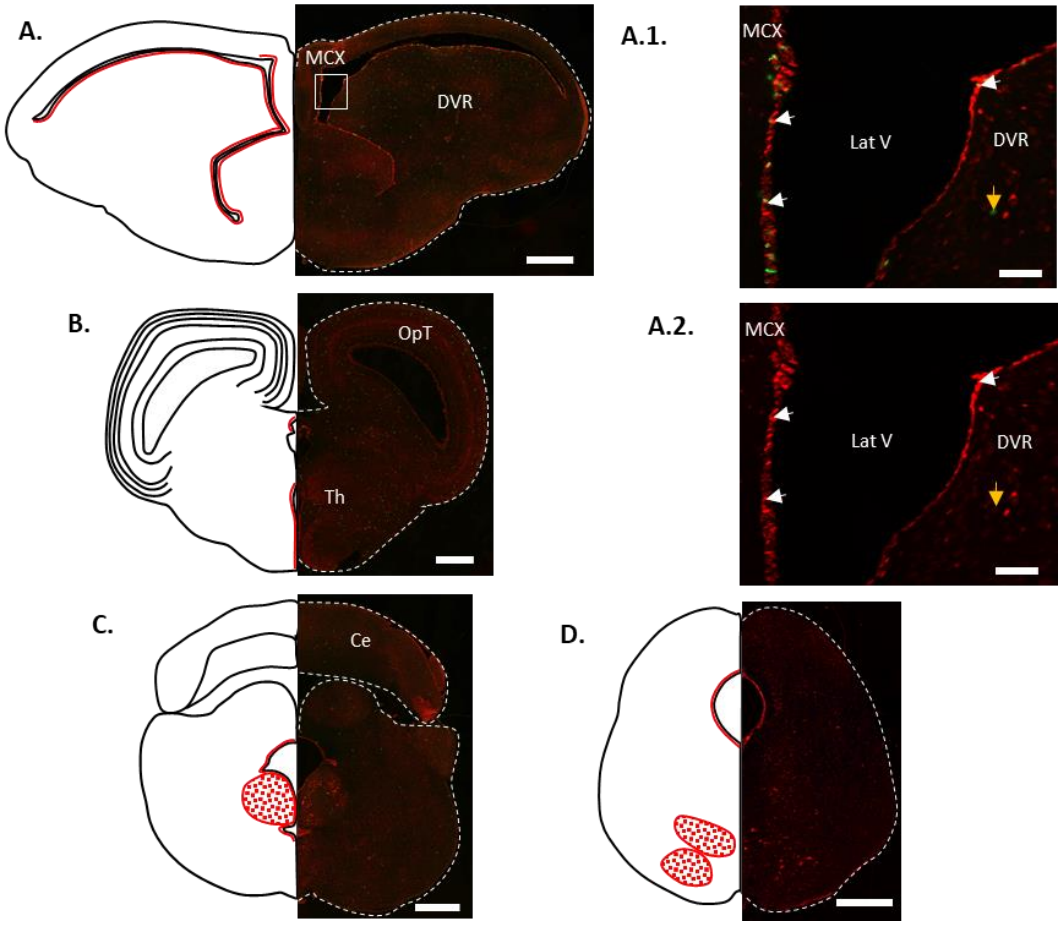


Figure 11. Sox2 expression pattern throughout the *Pogona vitticeps* brain at T1

A.-D. are split images where the left side is a diagram and the right side a fluorescence microscope image. The diagrams show distribution of Sox2 in the different brain regions shown in red. The fluorescence images show Sox2 in red and BrdU in green. A1.-A.2. are thumbnails showing a closeup of the boxed area in A. **A.** the telencephalon has Sox2+ cells in the lateral ventricular lining. **A.1.** The BrdU positive cells (green) in the lateral ventricular lining are also Sox2 positive (white arrows). The BrdU positive cells in the parenchyma are Sox2 negative (yellow arrow). **A.2.**The same cells that were BrdU positive in A.1., are also Sox2 positive (red) in this image (white arrows). The cell indicated by the yellow arrow is not Sox2 positive. **B.** Only the dorsal edge of the aqueduct and the edges between the hemispheres are Sox2 positive. **C.** The ventricular lining of the 3rd ventricle is Sox2 positive. The large cells in the oculomotor nucleus show as Sox2 positive. **D.** The ependyma of the spinal canal are Sox2 positive. The large cells of the motor nuclei also show up as Sox2 positive.

Scale bars: A.-D. 500µm, A.1.-A.2. 50µm

3.1.1.4. GFAP and Nestin expression pattern in Pogona vitticeps

GFAP positive glial cells were found throughout the brain in the surfaces adjacent to cerebrospinal fluid. These cells are glia limitans, and they play a role in regulating exchange between the CSF and brain tissue. These cells are mostly astrocyte-like in shape and can be found on the edges of all the sections except in the cerebellum (see figure 12). The GFAP+ cells lining the ventricles are more diverse in morphology, and do not cover all of the ventricular lining.

The lateral ventricular lining has the most interesting glial morphology. In addition to being a hotspot for regenerative ability, the lateral ventricular lining of the cortex has radial glia; this type of glial structure is prevalent during development. Radial glial progenitor cells are the embryonic stem cells that produce all neuronal and glial cell types in the brain. This type of progenitor cell is lost during maturation in mammals but persists into adulthood in many reptiles. The radial glial cells of the *Pogona* cortical area have end feet at both the ventricular lining and the pial lining, with a clear directional projection in between (see figure 12 A.1.). If these cells are also BrdU+, it would mean that our prospective stem cells are radial glia. This experiment does not clarify whether the radial glia are the same cells that are BrdU+ at one month, and an additional experiment with GFAP and BrdU together is needed to characterize these cells further. It is possible that the stem cells that persist after neurogenesis are a different type of progenitor, but that the same mechanisms that uphold the stem cell niche may facilitate the radial morphology in these glial cells. The fact that the ependyma adjacent to the DVR is totally devoid of GFAP+ cells, but still has putative stem cells, speaks to the theory that the radial glia and stem cells are different cell types. Alternatively, distinct progenitor populations with particular characteristics might exist in the different neurogenic niches, as previously shown in the telencephalon of fish (Ganz & Brand, 2016). The radial glia may also act as scaffolding to direct neuronal migration in the cortex when new neuronal cells find their way to their final destination.

Other areas in the telencephalon also have glia with radial projections. The striatal areas of the lateral ventricle also have GFAP+ glial projections radiating toward the

edge of the section, and there are projections from the glia limitans in the opposite direction. What differentiates these projections from the cortical radial glia is that they do not project all the way from the ventricular lining to the pial edge of the section. These cells, therefore, are not true radial glia but tanycites. These tanycites are on the opposite side of the lateral ventricle as most of the prospective stem cells in this area (compare figure 12 to figure 5).

Moving caudally through the brain slices we see the glia limitans as the predominant GFAP+ cell type outside the telencephalon. The only exception to this is the oculomotor nucleus, where there was heavy GFAP staining in the form of crisscrossing projections.

These slides were also stained with antibodies for Nestin, which is a stem cell marker, but also stains astrocytes. Unfortunately, only very few positive cells were noticed in the pogona brain, and no specific staining was observed in the stem cell niches. It is known that reptilian brains lack true astrocytes though astrocyte-like cells can be seen, so the lack of astrocyte staining cannot be used as an indicator for a faulty antibody. The specificity of this antibody still needs to be confirmed in reptile tissues. As the Nestin staining is inconclusive, it would have been better to pair BrdU with GFAP to find out whether the cortical radial glia are in fact putative stem cells.

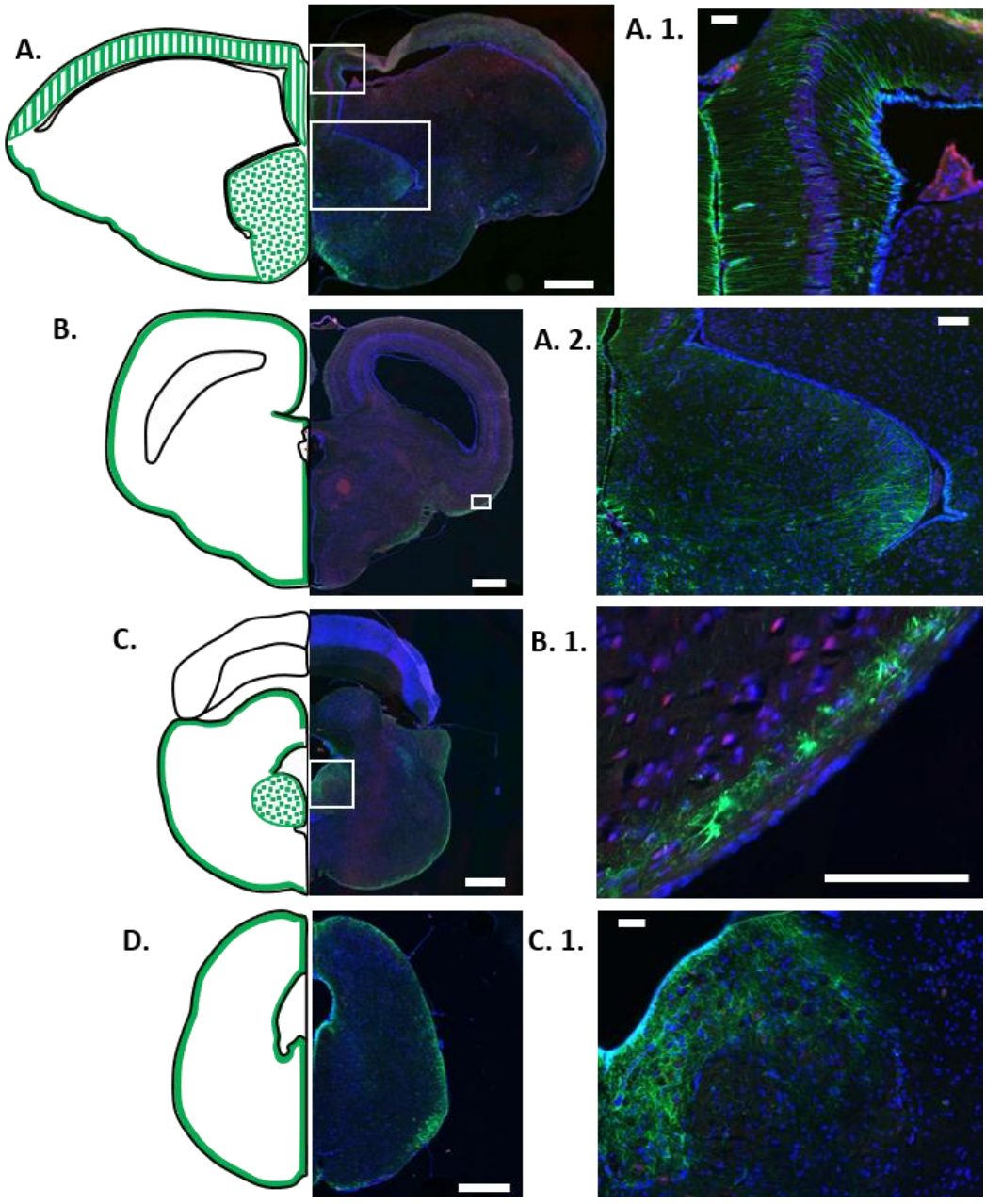


Figure 12. GFAP and Nestin expression pattern throughout the *Pogona vitticeps* brain

The left column is a split image where the left side is a diagram and the right side a fluorescence microscope image with GFAP in green and Nestin in red. The diagrams only show the distribution of GFAP in green. The lines around the edges represent glia limitans, the striped area represents radial glia, and the dotted areas represent GFAP+ cells that have no clear radial structure. The right column is enlarged portions of the fluorescence images on the left, shown as boxes in the original images. **A.** The most notable feature of the telencephalon is the radial glia throughout the cortical area. The striatum also has clear directional structure in the GFAP fibers, but it is not clear whether these cells have projections that span all the way from the lateral ventricle to the space between the hemispheres. Glia limitans can be seen surrounding the outer limits of the section. **A. 1.** An enlarged image of the top box in A. showing the structure of radial glia in the medial cortex. End feet can clearly be seen on the outer edge of the cortex and in the ependymal layer of the lateral ventricle. This structure persists throughout

the cortical area but becomes more disorganized the further it reaches laterally. **A. 2.** An enlarged image of the lower box in A. showing the striatum. Cells resembling radial glia can be seen radiating away from the lateral ventricle. The projections, however, do not seem to reach the limits of the hemisphere. **B.** Cross section of the hypothalamus and optic tectum. No radial structures, and only glia limitans can be seen as GFAP+. **B.1.** Closeup of glia limitans. Here the more astrocyte-like structure of glia limitans can be seen. It is clearly different from the end feet of radial glia. **C.** Metencephalon with cerebellum. Glia limitans surround the metencephalon, but not the cerebellum. The trochlear and oculomotor nuclei have GFAP+ projections. **C.1.** Closeup of GFAP+ projections in the trochlear and oculomotor nuclei. These projections are more interwoven than the structured radial glia in the cortical areas. They seem to have end feet in the 3rd ventricular lining, but only project throughout the nucleus. **D.** The myelencephalon lacks glial radial projections but has glia limitans.

Scale bars: A, B, C, D: 500 µm; A.2., B.1.: 100 µm; A.1., C.1. :50 µm.

3.1.1.5. HU expression pattern and neuronal differentiation in Pogona vitticeps

HU is a marker that can be seen in neuronal cells very early in the differentiation process. It is, therefore, a marker that can show the neuronal fate of BrdU+ cells even before they have fully differentiated into mature neurons. The images from T1 can shed light on whether the cells that divided at T0 (BrdU+) have taken on a neuronal fate. The cells that are double positive for BrdU and HU at T1 are neuroblasts that are derived from cells that divided at T0. The proliferation hotspots at T0 are the same as what has been shown in the preceding images, and therefore, these new neurons are derived from the proliferative hotspots shown in this thesis.

It is evident that in Pogona vitticeps a number of the BrdU labelled cells take on a neuronal fate (see figure 13). This is evident from the BrdU-HU double labelled cells that can be found surrounding the proliferative hot spots in the lateral ventricular lining. The stem cells in the lateral ventricular lining produce neuronal daughter cells, that migrate away from the ventricular lining. This can be seen in the telencephalon, where the areas adjacent to the lateral ventricular lining has the densest concentration of newly born neurons in the entire brain. Some new neurons can be found in the tegmentum of the mesencephalon and metencephalon, but the number or density do not rival that of the telencephalon.

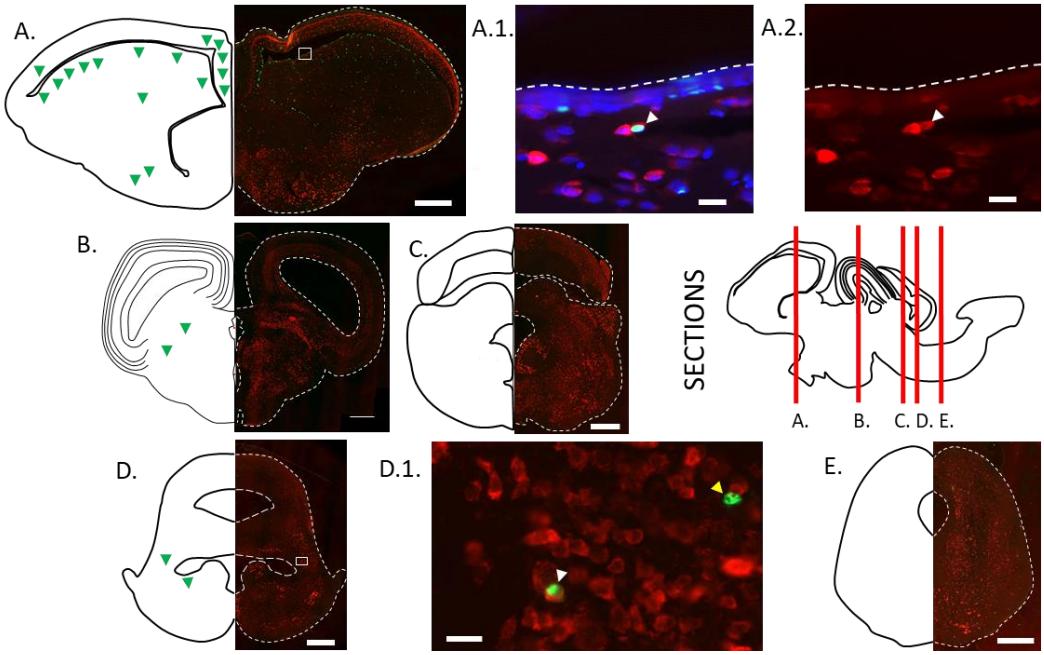


Figure 13. HU expression pattern throughout the *Pogona* brain at T1

The left side of each image is a diagram and the right side a fluorescence microscope image. The diagrams show distribution of all the double labelled cells found in all the studied brain slices as green triangles. The number and rough position are accurate for more than one slice throughout the region in question. The right side of each image shows a fluorescence microscope image with HU in red and BrdU in green. Double labelled cells are neuroblasts born from the cells that divided at T0. **A.** Shows the telencephalon with the densest population of newly born neurons in the entire brain. Most of these double labelled cells are found adjacent to the ventricular lining where they were born. **A.1.** A closeup of the ventricular lining and surrounding area marked in A. with a box. Here a double labeled cell can be seen (white arrow). HU is shown in red, BrdU in green, and DAPI in blue. **A.2.** The same image as A.1. showing only HU in red. It is clear from this image that the BrdU+ cell is also HU+ (white arrow). It can also be seen that the ventricular lining (adjacent to the dashed line) is not HU+, and therefore, not neuronal tissue. **B.** Shows the diencephalon and mesencephalon. Two new neurons were found close to the border between these areas. C. Depicts the metencephalon, where no new neurons were found. **D.** Shows two double labelled cells found adjacent to the third ventricle in the metencephalic tegmentum. **D.1.** Shows a closeup from D. (square) of two BrdU+ cells (green). One is also HU+ (white arrow) and the other is not (yellow arrow). **E.** Shows no double labelled cells in the myelencephalon.

Scale bars: 500µm for A.-E. and 20 µm for closeups A.1, A.2, and D.1.

The scattered BrdU+ cells throughout the parenchyma in *Pogona vitticeps*, however, do not exhibit neuronal markers. This observation leads me to believe that they may be glia and specifically polydendrocytes. These cells act as precursors for other types of glia and may have reactive regenerative properties for neuronal damage (Nishiyama et al., 2009).

The same areas that have large cells with high levels of Sox2 labelling also show up positive for HU. This observation confirms that these cells are neuronal, and the high levels of Sox2 do not denote stemness in these cells

3.1.2. *Pantherophis guttatus*

3.1.2.1. BrdU and PCNA in the different brain regions of Pantherophis guttatus

The main brain regions in *Pantherophis guttatus* are very similar to *Pogona vitticeps*. There are some anatomical differences between the species, but the overall brain structure has more similarities between these species than for example compared with mammals (see figure 2 for comparison, and figure 4 for the map of brain regions in *Pogona*). A comparison between constitutive neurogenesis in the same brain regions is, therefore, quite easily made between these species.

There is proliferation throughout the *Pantherophis* brain. Most of the proliferation can be seen in the telencephalon, where 78,6 % of PCNA+ proliferating cells reside at T1 and 76,4% at T0 (see figure14). The majority of proliferating cells are located in the lateral ventricular lining in a similar fashion to *Pogona vitticeps*. Unlike the *Pogona*, *Pantherophis* does not have the same level of proliferating cells throughout the parenchyma. Most of the proliferation seems to be confined to the proliferation hot spots that are similar to *Pogona*. A further comparison between the species will follow in a separate section, while the mapping of proliferative areas is discussed here.

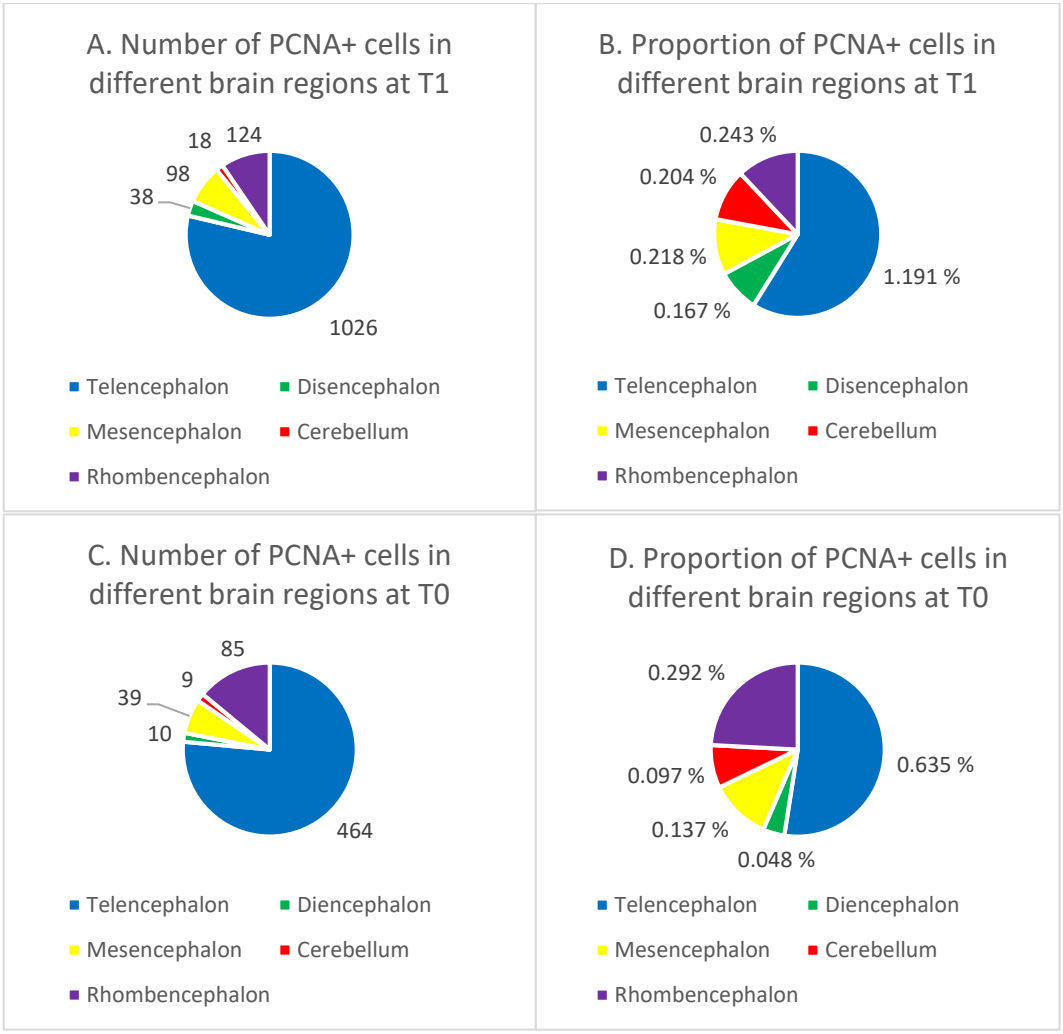


Figure 14. Proliferation in different brain regions in *Pantherophis guttatus*

A. The total number of PCNA positive cells in different brain regions at T1.

B. The proportion PCNA positive cells out of the full cell count of each region at T1.

C. The total number of PCNA positive cells in different brain regions at T0.

D. The proportion PCNA positive cells out of the full cell count of each region at T0.

There is less variation between the *Pantherophis* specimens than the *Pogona* specimens in this experiment. Most of the PCNA+ positive cells are clearly in the telencephalon. There they are confined to the lateral ventricular ependymal layer (see figure 15). None of the ependymal lining of the other ventricles exhibit proliferation, with the exception of the olfactory tract. These areas are linked by the RMS, and it is still unclear whether the cells are born in the olfactory tract, or whether they have divided from lateral ventricular stem cells and then migrated to the olfactory canal.

No putative stem cells were found in the section from the olfactory bulb, but it must be considered that there was only one representative section from this area, and the count of stem cells in this species was very low.

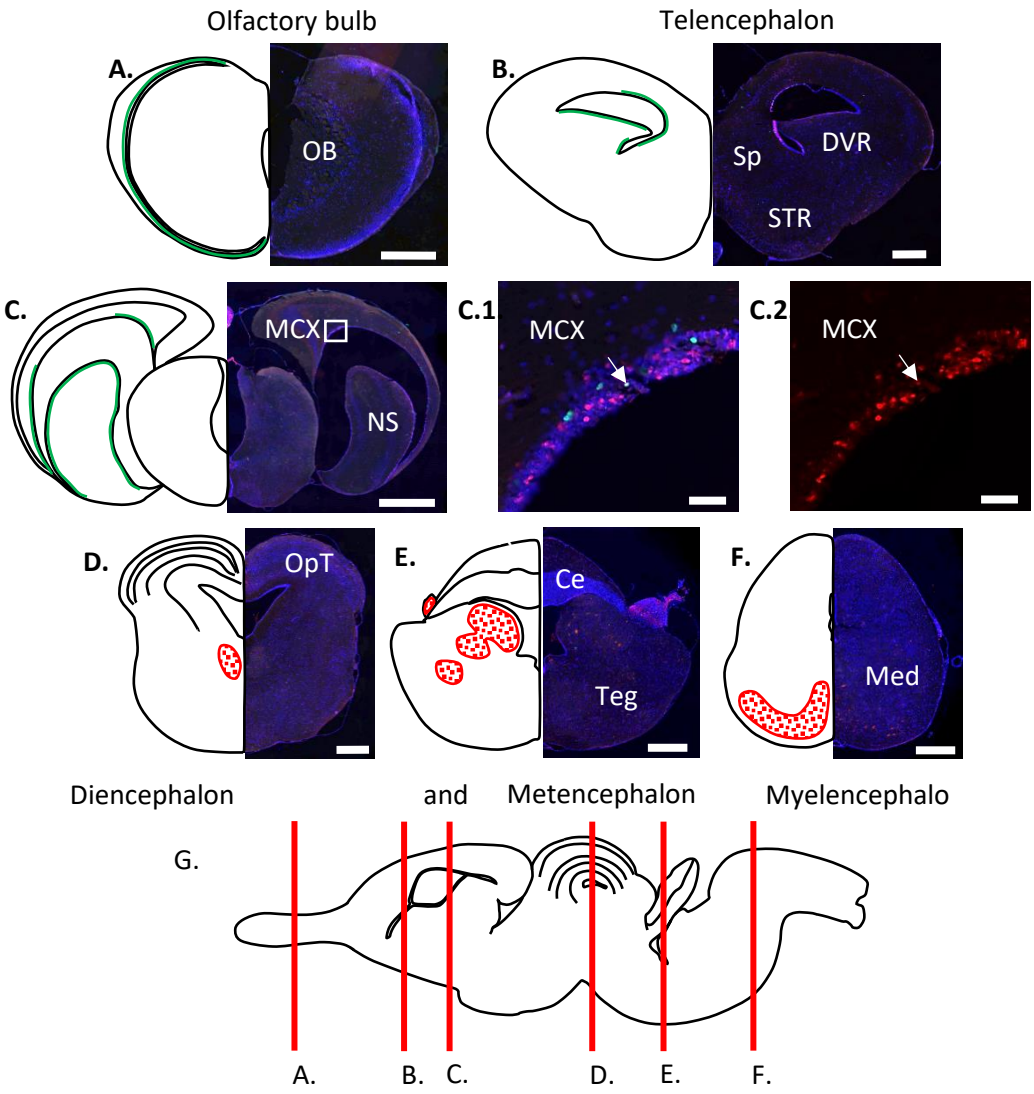


Figure 15. Proliferative areas throughout the *Pantherophis guttatus* brain at T1

Coronal sections showing the distribution of proliferative areas throughout the different major brain regions at T1. On the left of each panel a schematic drawing shows proliferative area in green along the ventricular lining. They also show non-specific PCNA labelling in red. The right side of each panel is a microscope image with DAPI in blue, BrdU in green, and PCNA shown in red. **A.** The olfactory bulb has proliferation in the lining of the lateral olfactory tract. **B.** Proliferative areas are confined to the ventricular lining of the lateral ventricles adjacent to the MCX and DVR. **C.** Proliferation is confined to the ventricular lining of the lateral ventricle adjacent to the MCX, LCX, and NS. **C.1.** Close up of the lateral ventricular lining seen in the white box in C. DAPI in blue shows all cell nuclei, PCNA in red shows

proliferation, and BrdU in green shows cells that divided at T0. The white arrow points to a stem cell that is double labelled with BrdU and PCNA. **C.2.** The same image as in C.1. but showing only PCNA in red. The same cell is indicated with an arrow as in C.1. where it is clearly seen as BrdU positive. Here we can see that it is also PCNA positive. **D.-F.** No clear proliferative zones can be seen outside of the telencephalon and olfactory bulb. All the ventricular lining is also devoid of proliferation. Areas showing large cells with non-specific PCNA labelling are shown in red. **G.** Sagittal section of the Pantherophis brain showing where each of the coronal sections (A.-E.) are from. Scale bars: A.-B. 500µm, C. 1000 µm, C.1.-C.2. 50µm, D.-F. 500 µm.

Only six BrdU-PCNA double labelled stem cells could be found in the telencephalon, and one double labelled cell in the diencephalon, with the rest of the brain completely devoid of stem cells (see figure 16). Though there is proliferation throughout the brain, the very few stem cells are strictly found in very specific small areas. Only 0,007% of the cells in the telencephalon are BrdU-PCNA double labelled, which is far less than what can be seen in *Pogona*.

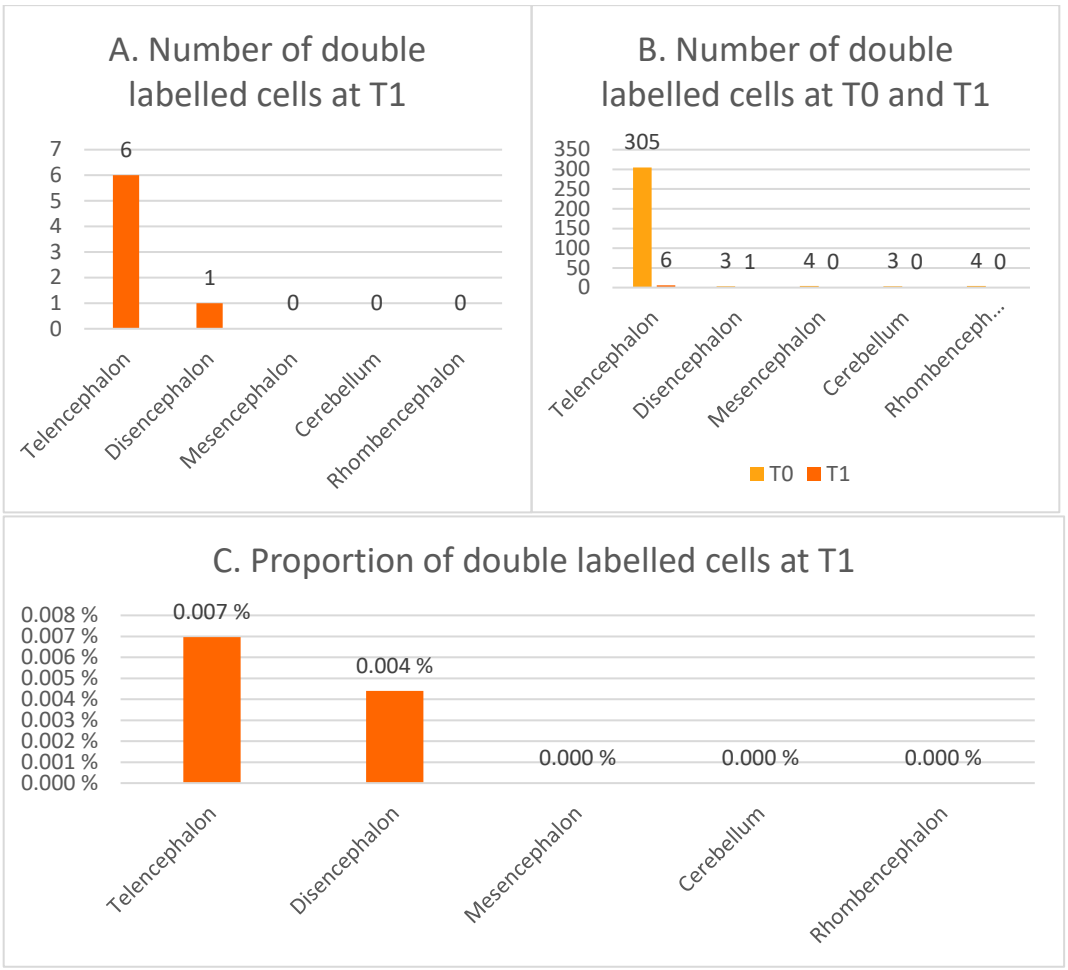


Figure 16. Distribution of possible stem cells in different brain regions in *Pantherophis guttatus*

This figure shows the number and proportion of cells that are double labelled with BrdU and PCNA in the different brain regions. These cells have divided at T0 and again at T1 and are prospective stem cells. **A)** How many double labelled cells were found in the different brain regions at T1, showing the number of possible stem cells in the different brain regions. The stem cells are mainly found in the telencephalon. **B)** The change of the number of double labelled cells from T0 to T1. At T0 the number of BrdU and PCNA double labelled cells indicates how much proliferation is happening in the different brain areas. At T1 we have the number of possible stem cells shown separately in A. **C)** The percentage of all cells in each brain region that are double labelled.

3.1.2.2. BrdU and PCNA in different regions of the Pantherophis guttatus telencephalon

The vast majority of putative stem cells are found in the lateral ventricular lining of the telencephalon. Proliferation can be seen in both the ependyma and parenchyma of the telencephalon, however, most of the proliferation is restricted to the ependymal layer, where the stem cells are located (see figure 17).

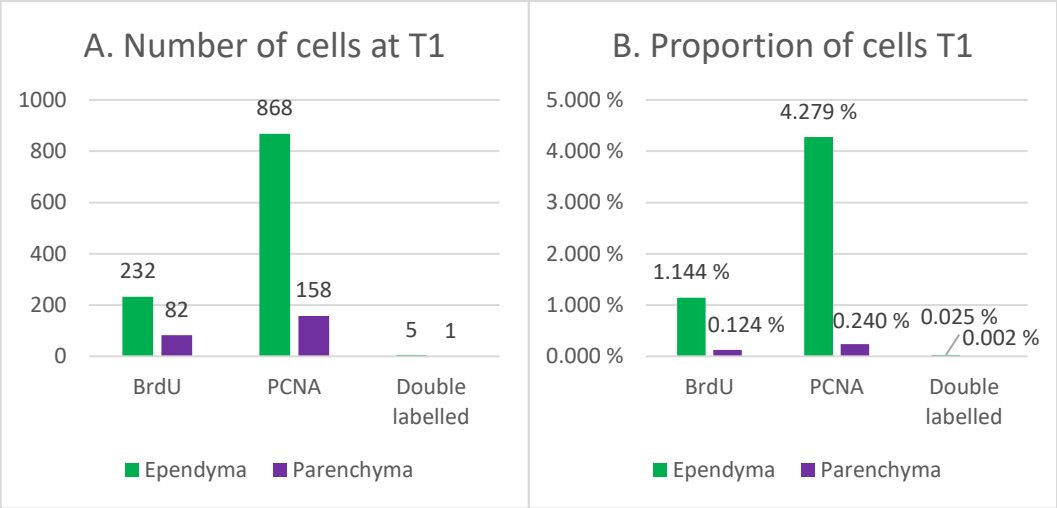


Figure 17. Proliferation pattern in the lateral ventricular ependyma compared to telencephalic parenchyma in *Pantherophis guttatus*

This figure shows the number (A.) and proportion (B.) of labelled cells from the full cell count of the lateral ventricular lining (green) compared to the telencephalic parenchyma (purple) at T1. BrdU shows proliferation at T0, PCNA shows proliferation at T1, and BrdU-PCNA double labelled cells show possible stem cells. Both the number and proportion of proliferating cells are higher in the ventricular ependyma than the surrounding parenchyma. All but one of the possible stem cells were found in the ventricular lining, with only 1 or 0.002 % in the surrounding parenchyma. The lateral ventricular lining is the proliferative hot spot also in the corn snake.

Though there is a smaller number and proportion of proliferating cells and putative stem cells in *Pantherophis*, the overall pattern of proliferative zones is similar to the pattern observed in *Pogona*. The MCX and DVR are proliferative hot spots. The DVR has areas of sparser proliferative activity in some sections. There are two notable differences in *Pantherophis*: both the NS and a section of DCX exhibit proliferation that is not evident in *Pogona*.

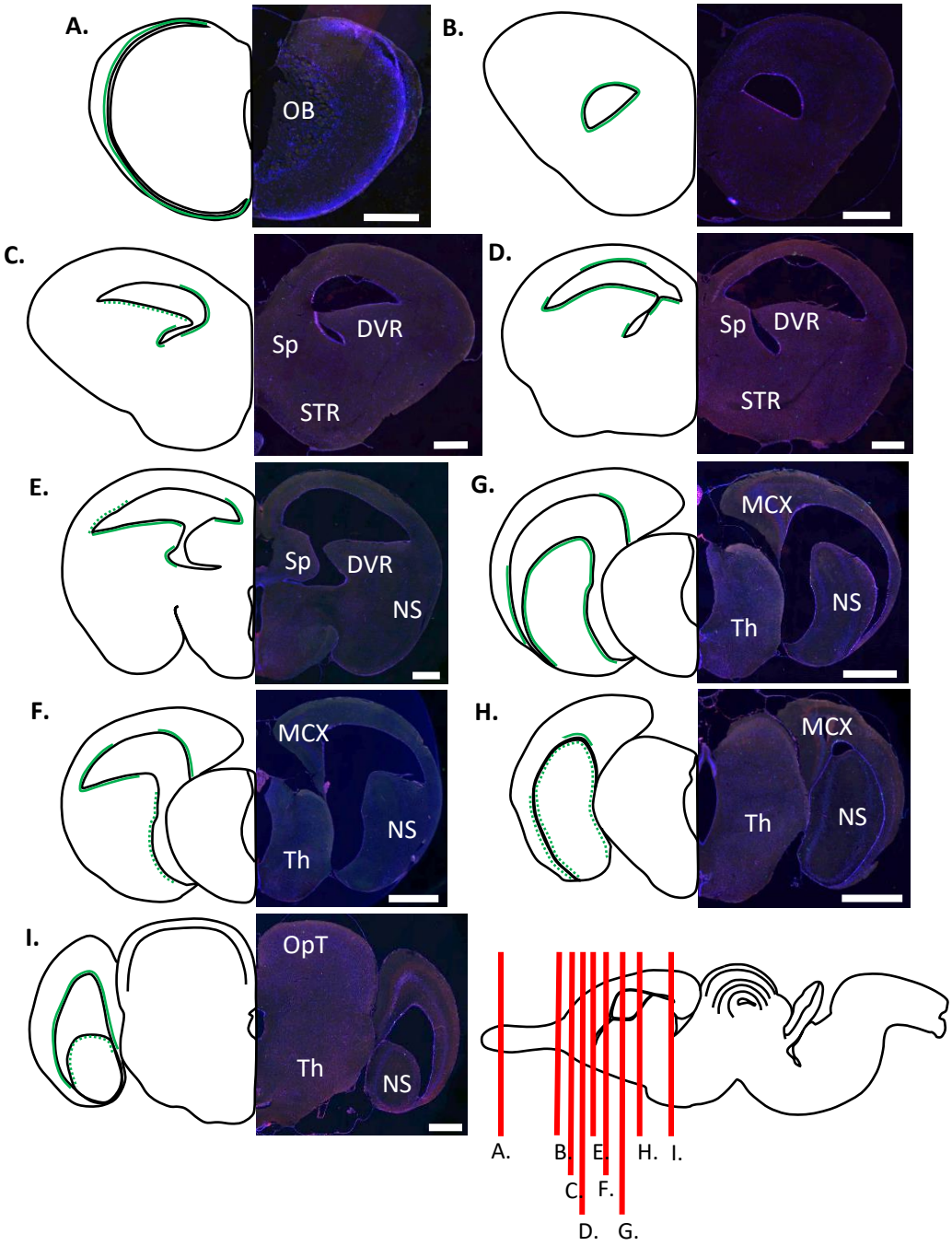


Figure 18. The lateral ventricular proliferative zones in *Pantherophis guttatus*

A.-I. are coronal sections of the telencephalon from anterior to posterior, as seen in the lower right corner. The red lines in this sagittal section indicate where each section is located in the telencephalon. Proliferative areas of the lateral ventricular ependyma are shown in green. **A.** The olfactory bulb has proliferative cells along the outer wall of the olfactory tract. **B.** the entire ventricular ependyma is proliferative in the rostral part of the lateral ventricle. **C.** The MCX and DVR ependymal areas are proliferative, with the dotted line indicating fewer proliferating cells. **D.** A short section of DCX has

proliferative cells. **E.** the MCX, DVR, and lateral ventricular tip are proliferative. **F.** The MCX and Lateral ventricular tip are proliferative. **G.-I.** The MCX and NS are proliferative.

Starting from the anterior telencephalon, proliferating cells can be found everywhere in the lateral ventricular lining, but as distinct brain regions begin to appear in the sections, the proliferative areas become clearly linked to them. The ependyma adjacent to the MCX, DVR, and NS is proliferative throughout the sections, and the DCX and LCX ependyma exhibit proliferation in some of the sections. The proliferation in the LCX ependyma always is linked to proliferation around the tip of the lateral ventricle, and persists to different degrees along the LCX.

3.1.2.3. Sox2 expression pattern in Pantherophis guttatus

Sox2 is found throughout the ventricular system of the brain, but especially in the areas where proliferation is abundant. The same lateral ventricular zones that are proliferative are additionally Sox2+, which is to be expected, as Sox2 is known for its ability to keep cells in a state permissive for replication. One interesting finding was that some of the non-proliferative areas of the ventricular lining were also positive for this marker (see figure19). These areas included a small area of the 3rd ventricle, the ependyma of the tectal ventricles, as well as the cerebellar lining adjacent to the molecular layer. No proliferation was seen in these areas even though Sox2 was present and the positive cells had direct contact with CSF from the ventricles, which is an important aspect of the stem cell niche.

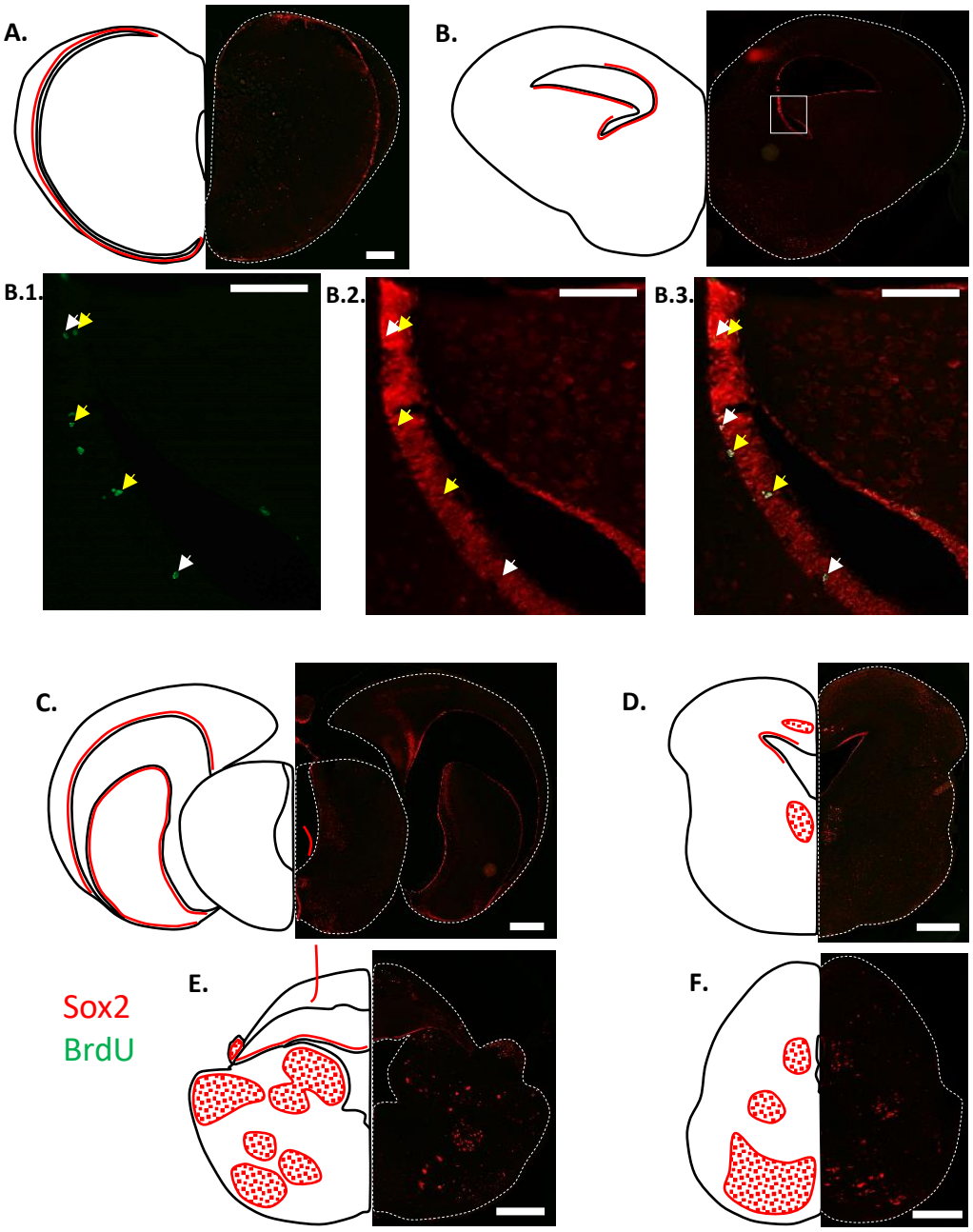


Figure 19. Sox2 throughout the *Pantherophis guttatus* brain at T1

The left half is a schematic drawing, with Sox2+ areas marked in red. The right half is a fluorescence microscope image, showing Sox2+ cells in red, and BrdU positive cells in green. **A.** The olfactory bulb with Sox2+ olfactory tract ependymal cells. **B.** The caudal part of the telencephalon showing Sox2+ cells in the lateral ventricular ependyma. **B.1.-B.3.** enlarged image of the boxed area in B. Here BrdU labelled cells can be seen in green, with Sox2+ cells in red. Many of the BrdU+ cells are also Sox2+ (yellow arrows), and some are only positive for BrdU (white arrows). **B.1.** BrdU+ cells in the lateral ventricular ependymal lining. **B.2.** Sox2+ cells in the lateral ventricular ependymal lining. **B.3.** BrdU+ cells (green) and Sox2+ cells (red) in the lateral ventricular ependymal lining, with some of the cells double labelled (yellow arrows). **C.** Caudal telencephalon with diencephalon. Sox2+ cells can be seen throughout the

lateral ventricular lining, in the dorsal part of the 3rd ventricle, and between the hemispheres. **D.** Mesencephalon with Sox2+ cells in the tectal ventricular lining, but not in the 3rd ventricular lining. Some non-specific staining of large cells can be seen in the oculomotor nucleus and the nucleus of the trigeminal nerve (marked with red dots in diagram). **E.** Metencephalon with Sox2+ cells in the ependyma adjacent to the cerebellar molecular layer. More non-specific staining can be seen in the large cells of the reticular and trigeminal nuclei. **F.** Rhombencephalon with only non-specific staining in large cells.

Scale bars: A: 200µm, B: 500µm, B.1-3: 50µm, C-F: 500µm

As in Pogona, the majority of the BrdU+ cells in the lateral ventricular lining are also Sox2+. The scattered BrdU+ cells in the parenchyma are not Sox2+. The images in figure 19 are from T1 to compare BrdU+ possible stem cells to the Sox2 staining. The images from T0 show a similar pattern for Sox2, but with more double labelled cells in the ependymal lining. Finding cells that are double labelled with Sox2 and BrdU at one month after BrdU treatment supports the hypothesis that the BrdU+ cells in the ependymal lining are truly stem cells.

3.1.2.4. GFAP and Nestin expression pattern in Pantherophis guttatus

The pattern of GFAP+ staining in *Pantherophis* is quite different from that of *Pogona*. The radial glia are missing entirely, and there are less tanycites with projections. Instead, wide swaths of astrocytes-like cells can be found in the parenchyma. Similar to *Pogona*, glia limitans are found throughout the brain, with the only exception in the olfactory bulb, which is completely devoid of GFAP+ cells.

The telencephalon of *Pantherophis* lacks the ordered radial structures in the cortical areas that are found in Pogona. Only two short stretches of the lateral ventricular ependyma are GFAP+. The first part is directly below the MCX. This is the only lateral ventricular area that has tanycyte projections, and they project into the MCX and septal area (see figure 20 B., B.1. and C.). The other ependymal GFAP+ area is at the tip of the ventral horn of the ventricle. There is a path from this point, through the striatum, to the periphery of the section that is densely populated astrocyte-like cells.

The star-like shape can clearly be seen in figure 20 B.2. The glia limitans is concentrated on the ventral half of the sections in the telencephalon.

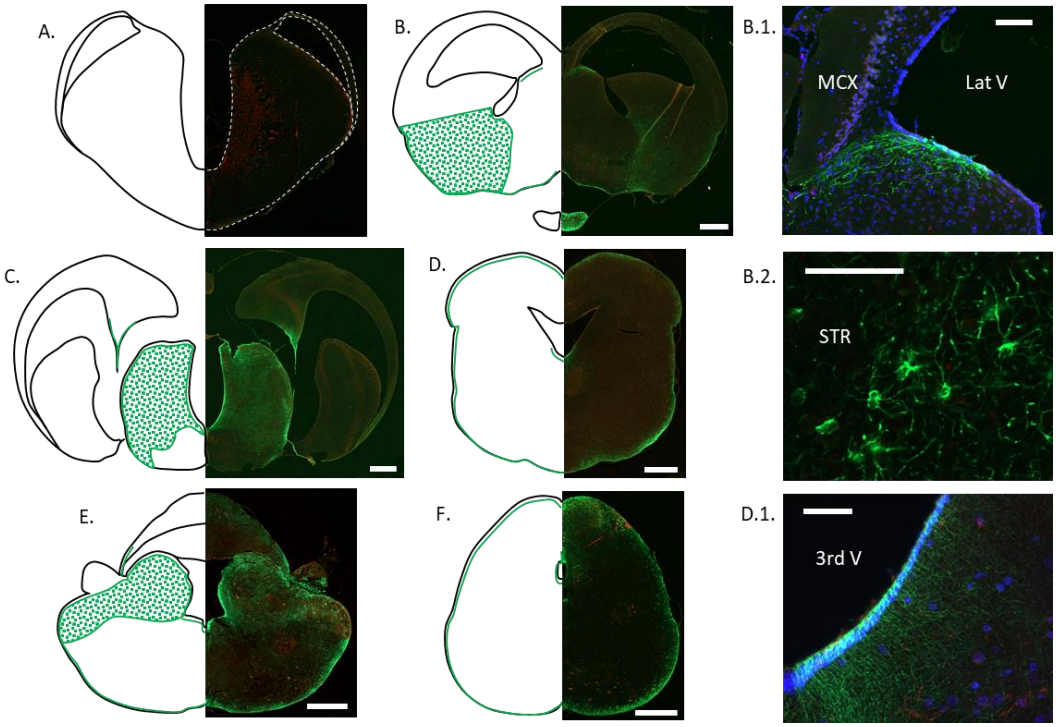


Figure 20. GFAP throughout the *Pantherophis guttatus* brain

The left half is a schematic drawing, with GFAP+ areas marked in green. The right half is a fluorescence microscope image, showing Nestin+ cells in red, and GFAP+ cells in green. **A.** The olfactory bulb has no GFAP+ cells. **B.** The telencephalon lacks radial glia in the cortex, but there is a short stretch of tanycyte projections from the ependymal layer below the MCX into the septum. A dense astroglial population can be found in the striatum **B.1.** a closeup of the tanycyte projections into the septum. **B.2.** A closeup of astrocytes, showing their star-like shape. **C.** The telencephalon with diencephalon A dense astroglial population can be found throughout the thalamus and hypothalamus. **D.** This area exhibits only glia limitans and tanycyte projections in the oculomotor nuclei. **D.1.** closeup of the oculomotor nucleus. **E.** Cerebellum and mesencephalon dense astroglial population in the top half of the metencephalon tegmentum. **F.** The rhombencephalon has only glia limitans, and a slight concentration of astrocytes in the dorsal area.

The rest of the brain has glia limitans throughout the section periphery. The most notable difference in comparison to Pogona is the densely astrocyte-like cell populated areas. These areas are the hypothalamus and the dorsal half of the metencephalon tegmentum (figure 20 C. and D. respectively). The third ventricular and aqueduct ependyma are GFAP+, and tanycyte projections can be seen projecting into the hypothalamus and the troclear and oculomotor nuclei.

3.1.2.5. HU expression pattern and neuronal differentiation in Pantherophis guttatus

HU is found in cells with a neuronal fate and is evident fairly early down this path of differentiation. It is, therefore, a good indicator for neurons that have been generated at T0. Figure 21 shows the distribution of newly formed neurons in the *Pantherophis guttatus* brain.

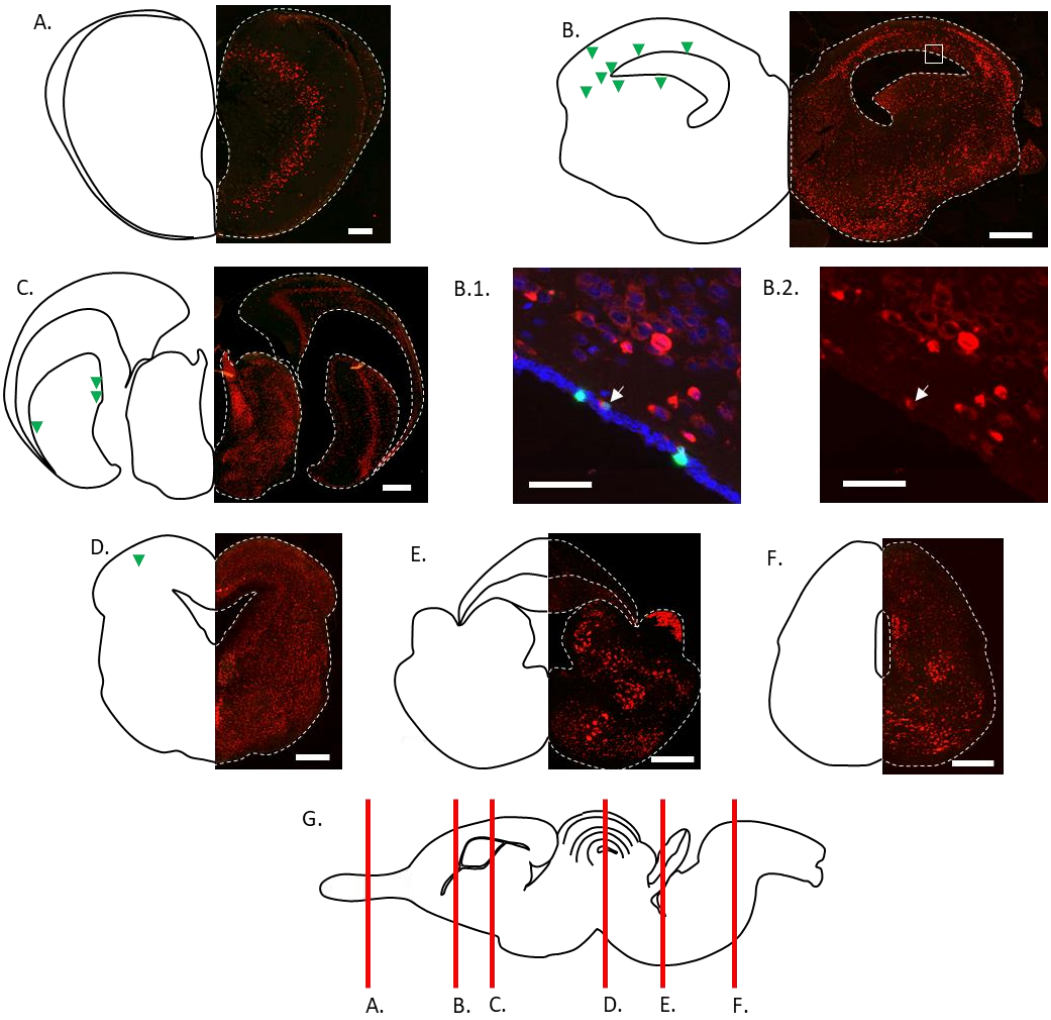


Figure 21 HU throughout the *Pantherophis guttatus* brain at T1

The left side of each image is a diagram and the right side a fluorescence microscope image. The diagrams show distribution of all the double labelled cells found in all the studied brain slices as green triangles. The number and rough position are accurate for more than one slice throughout the region

in question. The right side of each image shows a fluorescence microscope image with HU in red and BrdU in green. Double labelled cells are neuroblasts born from the cells that divided at T0. **A.** Shows the olfactory bulb, with no double labelled cells. **B.** Shows the telencephalon with the densest population of double labelled cells surrounding the lateral tip of the lateral ventricle. **B.1.** is a closeup of the ventricular lining area marked with a box in B. Here a double labeled cell can be seen (white arrow). HU is shown in red, BrdU in green, and DAPI in blue. **B.2.** The same image as A.1. showing only HU in red. It is clear from this image that the BrdU+ cell is also HU+ (white arrow). **C.** Shows the telencephalon and diencephalon. Double labelled cells can be seen in the nucleus sphericus. **D.** Depicts the mesencephalon, where one double labelled cell was found in the optic tectum. **E.** Shows the metencephalon with no double labelled cells. **F.** Shows the myelencephalon also with no double labelled cells.

Scale bars: 200µm for A., 500µm for B.-F. and 50 µm for closeups B.1 and B.2.

The newly born neurons come from the lateral ventricular lining. The BrdU positive cells in this region do not express the HU neuronal marker, and hence are not neuronal cells. Almost all of the double positive cells are found surrounding the lateral ventricles. This position makes sense, as the newly formed neuroblasts migrate from the lateral ventricular lining to the surrounding neural tissue to integrate. These findings confirm that the cells formed from non-neuronal stem cells in the lateral ventricular lining take on a neuronal fate.

3.2. Comparison between the bearded dragon and corn snake

3.2.1. BrdU and PCNA in different brain regions

The most obvious difference between *Pogona vitticeps* and *Pantherophis guttatus* is the density of putative stem cells in the brain. The difference can be seen by eye from the microscope images (compare figure 5 to figure 15), and our quantification indicates a near tenfold increase in the number of putative stem cells in *Pogona* when compared to Pantherophis (see figure 22). The stem cells, however, are situated in

similar regions in both species. The telencephalon has the majority of stem cells in both species, with only a few outliers found in other brain regions.

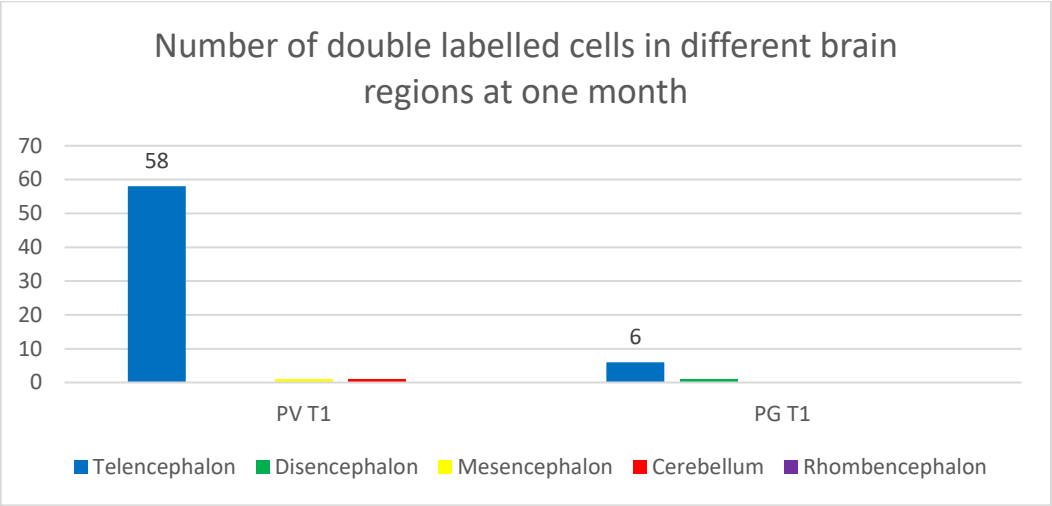


Figure 22. Number of stem cells in different brain regions in *Pogona vitticeps* and *Pantherophis guttatus* at T1

Almost all stem cells are found in the telencephalon in both species, with only a few outliers in other brain regions. There is an almost tenfold difference in the number of stem cells in the telencephalon between the species.

3.2.2. BrdU and PCNA in different regions of the lateral ventricles

The lateral ventricular lining is home to most of the dividing cells in both species. The proliferative hotspots in the ventricular lining are also situated in very similar regions between the species, though *Pantherophis* has far fewer stem cells than *Pogona*. As seen in figure 23, the areas adjacent to the MCX, DVR, and the lateral tip of the ventricle are proliferative. The main difference is that *Pantherophis* has a proliferative area below the DCX, which does not show up in *Pogona* (see figures 10 and 18 for comparison).

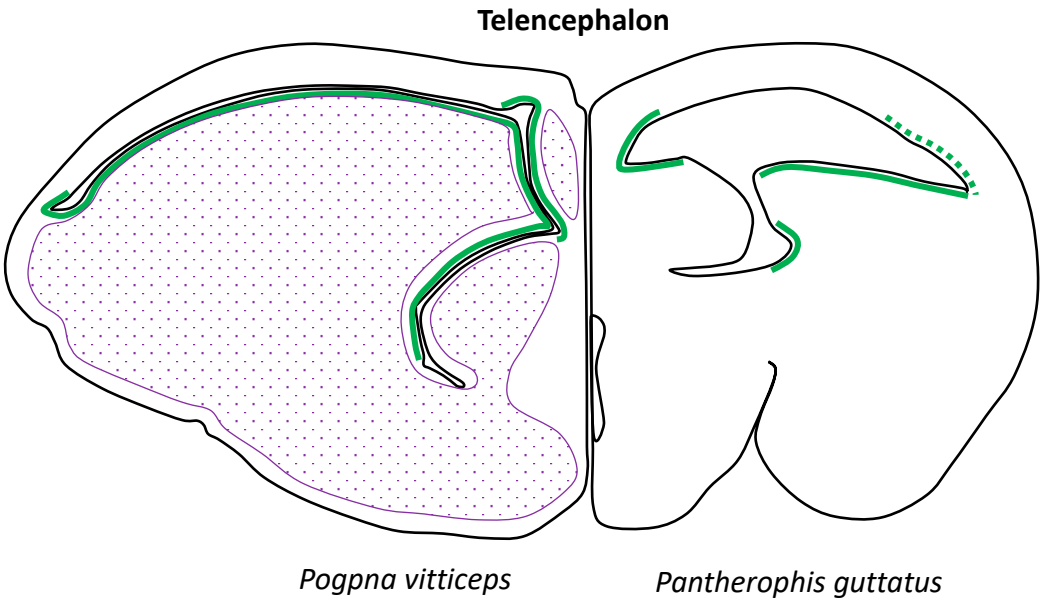


Figure 23. Comparison of proliferating areas between the *Pogona vitticeps* and *Pantherophis guttatus* telencephalon

Both species have proliferative zones (green line) surrounding the DVR and along the MCX in the ventricular lining. *Pogona vitticeps* (left) has additional dividing cells throughout the parenchyma (purple dotted area). *Pantherophis guttatus* (right) does not have these scattered dividing cells.

Pogona has scattered proliferating cells throughout the parenchyma, which are completely missing from *Pantherophis*. These cells may be polydendrocytes, which represent a resident glial progenitor cell population, and may play a part in reactive regeneration.

The BrdU and PCNA double labelled potential stem cells are found in the lateral ventricular lining in both species. *Pogona* has a higher number of these stem cells than *Pantherophis*, with 58 cells in the lateral ventricular lining, as opposed to 6 cells in *Pantherophis*. This disparity is seen not only in the number but also in the density of the putative stem cells, with 0,369% of lateral ventricular ependymal cells double positive in *Pogona* and only 0,025% in *Pantherophis* (see figures 8 and 17 respectively). The areas of the ependyma where stem cells are found is more widespread in *Pogona* than *Pantherophis*, with cells found throughout the ependymal lining, except adjacent to the dorsal cortex. In *Pantherophis* these stem cells are only found in the lining adjacent to the medial cortex. The density of cells in the medial cortex ependyma is

higher in *Pogona* (1,222%) than in *Pantherophis* (0,073%). Thus, both the number and density of stem cells is Higher in *Pogona*, and the stem cells are found in a wider area of the ependyma.

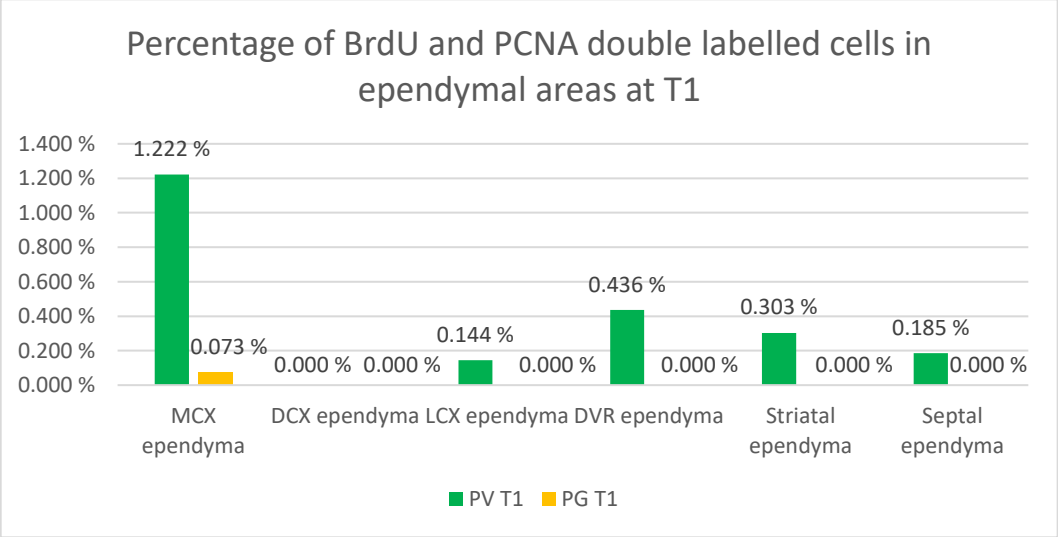


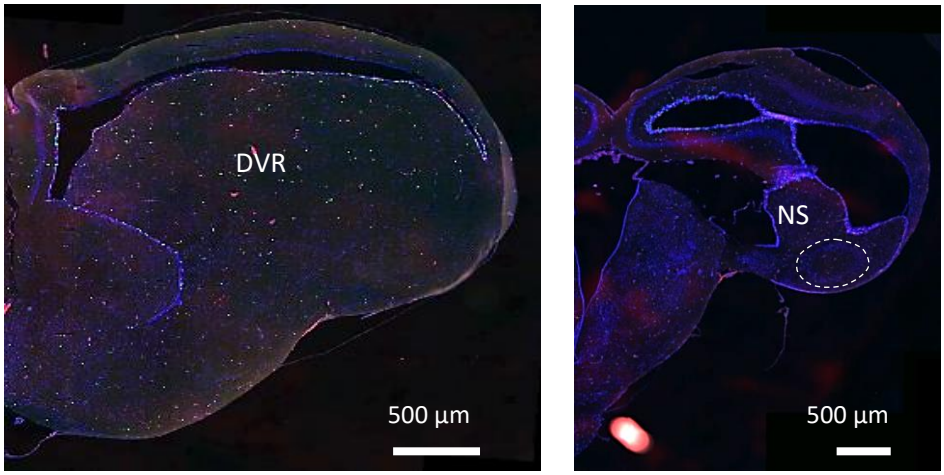
Figure 24. Location and density of stem cells in *Pogona vitticeps* and *Pantherophis guttatus*

This figure shows the percentages of BrdU and PCNA double labelled cells from the cell count of the ependymal layer. *Pantherophis* (orange) has stem cells only in the ependymal lining adjacent to the medial cortex. *Pogona* (green) in contrast has stem cells throughout the lateral ventricular lining, except in the area adjacent to the dorsal cortex. The medial cortex has the highest density of stem cells in both species.

There is one notable anatomical difference between the species in the telencephalon: the nucleus sphericus (NS) is much more pronounced in *Pantherophis* than *Pogona* (see figure 25). It is much larger in *Pantherophis* and comes right up to the lateral ventricular lining, making it a significant anatomical difference for this experiment. In *Pogona* the small nucleus sphericus remains underneath the DVR in the caudal regions of the telencephalon, never reaching the ventricular lining. In *Pantherophis*, however, the NS is much larger, and takes up a vast area directly adjacent to the proliferative ventricular lining in the mid and caudal sections of the telencephalon. The DVR in *Pantherophis* is much smaller than in *Pogona* and gives way to the NS as the telencephalon begins to overlap sections of the thalamus. The reason for this difference in brain anatomy is speculatively due to snakes being reliant on olfaction

for survival. Tracts from the olfactory bulb terminate in the LCX and tracts from the accessory olfactory tract terminate in the NS (Font et al., 2002; Iwahori et al., 1995). It would be logical in an evolutionary context for snakes to have a large NS to process the important olfactory information, in contrast, lizards tend to rely more on sight. The DVR processes three important sensory modalities: visual, auditory, and somatosensory (Ten Donkelaar, 1998). The volume taken up in the brain by these senses reflects the animal's behavior and capabilities in the wild (Lisney et al., 2007). Both the NS and DVR are areas that deal with sensory information, which has been proposed to be a major reason for brain regenerative capabilities in animals that grow throughout their lives (Alunni & Bally-Cuif, 2016).

Pogona vitticeps



Pantheophis guttatus

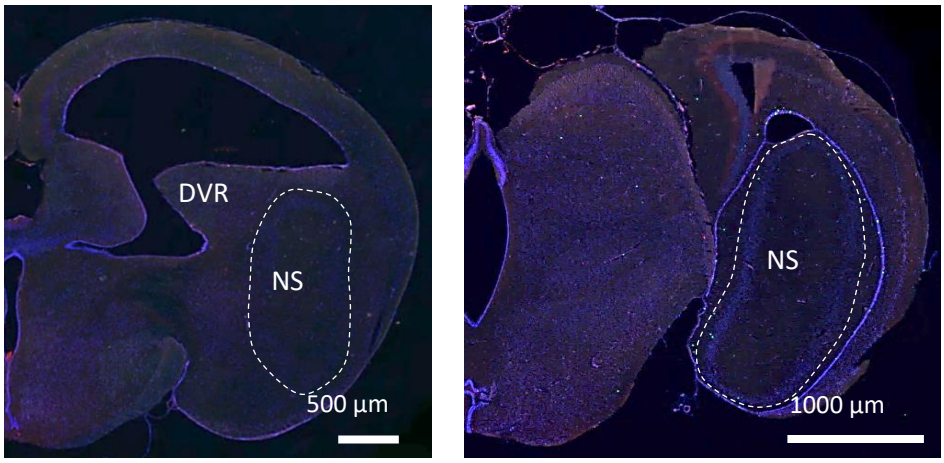


Figure 25. DVR and NS in *Pogona vitticeps* and *Pantherophis guttatus*

On the top row *Pogona vitticeps* exhibiting the DVR next to the ventricular lining (left) and the NS small and separated from the ventricular lining (dashed line). On the bottom row *Pantherophis guttatus* with

the DVR adjacent to the ventricular lining in the more rostral areas (left), and as more caudal sections are reached, the NS (dashed line) takes over this area below the lateral ventricle (right). DAPI in blue, BrdU in green, and PCNA in red.

In addition to the different sensory needs of the species the physical connection to the lateral ventricles is the key to proliferative activity in the *Pantherophis* NS. Proximity to the lateral ventricles seems to have a permissive influence on the stem cell niche. The NS only exhibits the same scattered BrdU positive cells found throughout the parenchyma in *Pogona*, but in *Pantherophis* proliferating cells can be found in the ventricular lining surrounding it, and BrdU positive cells are can be seen in the periphery. This difference is because the NS is adjacent to the ventricle in *Pantherophis* but not in *Pogona*. It may be that both a certain brain area with its specific signaling molecules and contact to the ventricle are needed to create areas permissive for stem cells to keep their stemness.

3.2.3. Comparison between species for Sox2, GFAP, and HU

Much of the ependymal lining in both species is Sox2+ (compare figures 11 and 19). The Sox2- regions in both species include the dorsal part of the lateral ventricle, and in *Pogona* the tectal ventricular lining. In *Pantherophis*, however, the tectal ependyma is Sox2+, and the 3rd ventricle as well as all ependymal areas caudal to it are Sox2-. All of the proliferative areas were also Sox2+ in both species.

Different areas in the two species are GFAP positive. In *Pogona* GFAP+ glia limitans can be found surrounding all brain areas except the cerebellum. In contrast, glia limitans are missing from the olfactory bulb and dorsal part of the telencephalon, as well as the cerebellum in *Pantherophis*. Different areas of the brain have tanycyte projections and astrocyte-like GFAP+ cells in the two species (compare figures 12 and 20). The most interesting finding is that *Pogona* has radial glia emanating from the lateral ventricles to the dorsal perimeter of the medial, dorsal, and lateral cortices.

This structure is missing in *Pantherophis*, and only the medial cortex had tanycyte projections and possibly radial glia in some areas of the MCX.

The cells that divided at T0 produced neuroblasts in both species, as seen by BrdU and HU double labelling (compare figures 13 and 21). All the neuroblasts were located near the lateral ventricles, except *Pogona* had two cells below the tectum. The neuroblasts were clustered around the lateral end of the lateral ventricles in *Pantherophis*, even though many of the prospective stem cells were located by the medial cortex. Three neuroblasts were also found in the nucleus sphericus in *Pantherophis*. There were more newly formed neuroblasts in *Pogona*, and they were more widely spread along the periphery of the lateral ventricles. The neuroblasts were found all along the DVR, but the density was larger toward the lateral tip of the lateral ventricles in *Pogona*. Another cluster of neuroblasts were found in the medial cortex in *Pogona*, which were completely missing in *Pantherophis*. The absence of these newly formed neurons in the *Pantherophis* MCX is very interesting, as most regenerative studies have centered on MCX regeneration.

4. Discussion

The aims of this study were to (1) map out the putative stem cell niches in *Pogona vitticeps* and *Pantherophis guttatus*, and (2) to compare the putative stem cell location and density between the species during constitutive neurogenesis. This experiment yielded answers to both of these questions. It was possible to map out the main brain regions in these new species using brain atlases of similar species, such as the gecko and tawny dragon among others (Hoops et al., 2018; Nieuwenhuys et al., 1998; Smeets et al., 1986). It was also possible to identify specific BrdU labelled cells as putative stem cells and show that daughter cells from these stem cells mature into neurons.

The lateral ventricular ependyma seems to be the main putative stem cell niche active in the normal state in both species. The BrdU pulse study showed that some of the cells in the ependyma along the MCX, DVR, and tip of the lateral ventricle had divided at T0 and were dividing again one month later at T1. These cells correspond to putative

stem cells, as they self-renew and are able to divide multiple times, and this theory is supported by the stainings with other markers. The ependymal cells of all of these areas are both Sox2 and GFAP positive, indicating that the stem cells are glial ependymal cells, and they have high levels of a transcription factor that maintains pluripotency. It was possible to show that the putative stem cells in these areas produced neuroblasts. Some of the cells close by these proliferative zones had divided at T0 (BrdU+), had migrated a short distance away from the ventricular lining by T1, and were positive for an early neuronal markers (HU) at T1. This observed neuronal fate of daughter cells from the ependymal lining is supported by previous studies (C. Lopez-Garcia et al., 1988; Pérez-Cañellas & García-Verdugo, 1996). The ependyma of the MCX, DVR, and tip of the lateral ventricle were the only areas in either of our species where all these factors came together, further indicating putative stem cell niches.

It has to be noted that there was some non-specific staining in the trochlear and oculomotor nuclei as well as the reticular system. All of these areas have large neurons (Newman & Cruce, 1982), which seem to have taken up the antibodies indiscriminately. They were also positive for HU, which is a neuronal marker, and should not show up in dividing cells. These cells show positive with all the antibodies, so the staining in them was deemed non-specific.

The most obvious difference in proliferation is that there were more active stem cells in *Pogona* than *Pantherophis*, and the proportion of these cells was also higher in *Pogona*. This difference in cell division can either be due to a smaller number of stem cells in *Pantherophis* or due to a lower amount of stem cells being active at any given time. The BrdU treatment was administered during the course of one week, with the goal of labelling all proliferative cells, including slowly cycling stem cells. A longer period of BrdU treatment may be needed to see whether a similar pool of stem cells cycle at a slower frequency in this species. It is likely that the number of cells is smaller in *Pantherophis*, but further testing is needed to confirm this theory.

Another clear difference between the species is that *Pogona* has radial glia in the medial, dorsal, and lateral cortices, when *Pantherophis* has tanycyte projections or possibly radial glia only in the medial cortex. It is unclear whether the projections span

the full length of the cortex, as the projections dim towards the central cell layer of the cortex. Differential findings in GFAP positive projections between reptile species has been found (Ahboucha et al., 2003; Lôrincz & Kálmán, 2015), with some species exhibiting highly ordered radial glia in the MCX and DCX, some with radial glia only in the MCX, and some only possessing radially oriented tanycyte projections in cortical areas. According to Lôrincz & Kálmán (2015) these projecting cells are radial trans-pallial projections in both snake and lizard patterns of GFAP expression, and their findings support the patterns seen in tis experiment. The only difference was the optic tectum, which was rich in GFAP positive radial cells in most of the species studied, and these cells were found in neither of the species in this study. The different patterns of GFAP immunoreactivity are a promising avenue in regenerative research, as they are actively involved in the removal of cellular debris after brain damage (López-García, 1999), and this function is carried out by the astroglia that cause glial scarring in mammals. Additionally, there is evidence that GFAP-expressing radial glia are putative stem cells in *Podarcis hispanica* (Font et al., 1995), and further experiments should include pairing BrdU and GFAP immunochemistry to ascertain whether this is the case in our species. Pairing GFAP with Nestin instead of BrdU was a missed opportunity in this experiment.

There were more newly formed neuroblasts in *Pogona* than *Pantherophis*, further indicating higher regenerative capacity under normal physiological conditions. The majority of the neuroblasts were found in the MCX and DVR in Pogona, which correlate to the putative stem cell niches identified in the ventricular lining adjacent to these areas. In *Pantherophis*, however, the neuroblasts were scattered widely around the tip of the lateral ventricle in a much less ordered fashion, and the newly formed neurons were completely absent from the MCX. There were also a few neuroblasts in close proximity of the ependymal lining in the NS. No putative stem cells were found in this region, but it is possible that these cells cycle so slowly that the BrdU pulse study did not catch them. There is a higher probability that the NS ependyma has putative stem cells that produced these new neuroblasts, than that they would have migrated from further away. This theory is supported by a study completed on *Podarcis hispanica*, where stem cells in the NS ependyma produced neurons that integrated into the mural layer of the NS (Perez-Sanchez et al., 1989).

Further experiments with longer BrdU pulses may uncover the existence of putative stem cells in the ventricular lining surrounding the NS. The presence of these neuroblasts in the *Pantherophis* NS also indicates that being adjacent to the ventricle is an important attribute of the stem cell niche. The NS in *Pogona* is further away from the ventricle and is devoid of both putative stem cells and newly formed neuroblasts, even though it has cells that are BrdU positive. There is a high probability that there are stem cells in the *Pantherophis* NS ventricular lining due to the NS being adjacent to the ventricle as well as there being evidence of newly formed neuroblasts in the structure. Previous studies have shown that stem cells in the ventricular lining have a single cilium that is in contact with the cerebrospinal fluid in the ventricles (García-Verdugo et al., 2002). Contact to the cerebrospinal fluid seems to be key in maintaining the stem cell niche also for the two species studied here.

The scattered BrdU positive cells that were found in the NS as well as throughout the parenchyma of the *Pogona* brain are of unclear origin. They do not come across as stem cells, as they do not show up double positive for BrdU and PCNA at T1. They also do not seem to produce neuroblasts, as the newly formed neuroblasts in *Pogona* are at a fairly uniform maximum distance from the ventricular lining. If these mystery cells had produced neuroblasts, the newly formed cells would most likely be as dispersed as their progenitors. These scattered BrdU positive cells are missing in *Pantherophis*. One theory is that they may be polydendrocytes, which can be found in the mammalian central nervous system. These cells are precursors to myelinating polydendrocytes but may also be involved in reactive neuronal regeneration (Nishiyama et al., 2009). Their presence in *Pogona* and absence in *Pantherophis*, however, is perplexing if this is the case. Our scattered BrdU positive parenchymal cells seem to be GFAP negative, which does not support the polydendrocyte theory, as glial cells should be GFAP positive (Dimou et al., 2008). Further studies are needed to characterize these cells and see if they indeed play a role in reactive regeneration in *Pogona*. Polydendrocytes are also known as NG2 cells after the neural/glial antigen 2 presenting proteoglycan found in its plasma membrane, and using a marker for NG2 together with BrdU would shed light on whether the BrdU positive cells in the *Pogona* parenchyma are in fact polydedrocytes.

The number of animals used in this pilot study was extremely small. These are new species to research, and that is why it was important to see whether they conform to the same patterns seen in other reptiles. Differences between lizards and snakes have not thus far been assessed. This small number of individuals is enough to show that the principal sight of constitutive neurogenesis appears to be similar to that of other reptiles, in spite of the apparent difference in number of proliferating cells, but new brain areas with relatively lower proliferative activity were also identified in this study. Furthermore, noticeable differences in the pattern of constitutive neurogenesis were observed between these lizard and snake species. It has to be noted that further studies with larger samples are needed to confirm our preliminary results and for detailed comparison between these species.

Further studies are needed to shed light on the differences between these species and what is behind their disparity in regenerative ability. It would be interesting to find out whether the scattered BrdU positive cells in the parenchyma truly are polydendrocytes, and whether they activate after brain insult to produce neurons. Another inviting avenue of study is focusing on the MCX, where *Pogona* has newly formed neuroblasts, and *Pantherophis* does not. This groundwork lays the way for further studies in regeneration in these two new species. Future studies comparing these species can bring forth new information, and these results may eventually be transferred to human brain research. Hopefully these future studies could lead to medications for brain disease and injury in humans.

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